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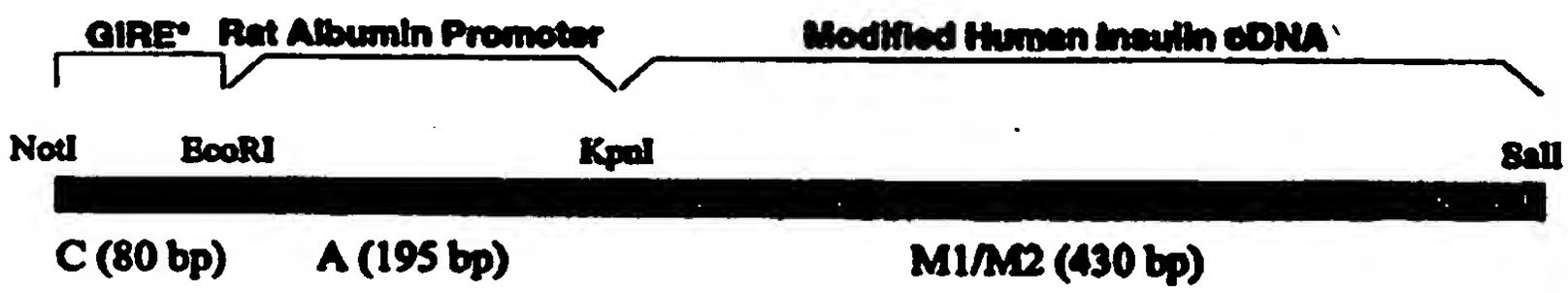

(57) Abstract

Hepatocytes transfected with a replication deficient adenovirus vector containing a gene cassette expressing the proinsulin gene in response to physiological levels of glucose provide novel beta islet replacement cells. The cassette comprises the structural gene for human proinsulin genetically altered to make it cleavable to active insulin, a promoter operably linked to the proinsulin gene, and a glucose regulatory response module located 5' to the promoter. Synthesis of proinsulin mRNA is ablated at less than 5mM glucose, and peaks at about 15 mM.

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TREATMENT OF DIABETES WITH SYNTHETIC BETA CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of co-pending application Serial Number 08/786,625 filed January 21, 1997.

FIELD OF THE INVENTION

This invention relates to the field of gene therapy and to a method of utilizing normal non-islet cells transfected with a proinsulin gene inducibly expressed in such cells in the presence of glucose. The proinsulin synthesized in the cells is further processed into mature insulin.

BACKGROUND OF THE INVENTION

Insulin-dependent diabetes mellitus (IDDM or Type I diabetes) occurs when an autoimmune response destroys the beta cells of the islets of Langerhans, resulting in cessation of insulin production. For many years, and indeed for many patients even at present, the only recourse for treating this fatal condition is the periodic administration of injectable insulin of animal, or more recently, of recombinant human origin. While the administration of exogenous insulin is life-saving over the long term, severe side effects, such as circulatory disturbances resulting in blindness, gangrene, and heart attack are common. The doses of insulin injected into the diabetic patient are only approximate, even when careful dietary controls are implemented. These continual imbalances in blood glucose resulting from deviations from optimal levels of insulin are thought to cause, or contribute to, the observed side effects.

There have been many attempts to minimize the side effects of insulin therapy. Home blood and urine glucose test kits help the diabetic monitor blood sugar

levels. Some researchers have proposed pump devices which meter insulin continuously and in more precise doses. One interesting such device is disclosed in U.S. Patent No. 5,364,838 which delivers insulin in aerosol form. The hormone is absorbed through the lung, thereby avoiding the more invasive injection route of administration.

In the field of diabetes there is a general recognition that replacement of beta cellular function would be a superior therapy to insulin administration, because the natural cell would secrete insulin in response to glucose levels in the microenvironment. This fine-tuned control would thereby eliminate the deleterious side effects of insulin administration. Indeed, it has been shown in the relatively small group of patients who have successfully received pancreatic transplants that remission of side effects or cessation of progressive tissue damage results. Unfortunately, pancreatic transplant is available only to a few diabetics, compared to the numbers of afflicted persons. In addition, transplantation is associated with significant toxicity due to immunosuppressive therapy.

There have been many alternatives proposed for providing the benefits of beta cell replacement without involving organ transplantation. These alternatives involve replacement of beta cell function with actual beta cells or other insulin secreting pancreas-derived cell lines, as discussed in Lacy, et al., Ann. Rev. Med., 37:33 (1986). Since introduction of exogenous cells into the body is perceived by the immune system as any other allograft, it is necessary to isolate the cells from contact with immunoactive cells and substances. In particular, donor islet cells must be protected from T-cells and macrophages mediating cytolytic processes. One approach is physical immunoisolation, either by microencapsulation or by a microporous chamber. The challenge in these

technologies is to overcome the natural process of foreign body rejection resulting in walling off the implant by a dense layer of fibroblasts. The key to a successful implant is to provide both a biocompatible surface and sufficient porosity to permit some degree of microvascularization. For a general review of these approaches, see Brauker, et al., J. Biomed. Mat. Res., 29: No. 12, 1995. To date, immunoisolation still poses significant problems including loss of viability and function of captive cell populations, foreign body rejection, fissure and inflammation, and immune reactions.

Another approach is to engineer beta cell function into cells derived from autologous tissue or artificially constructed cell lines. U.S. Patent No. 5,427,940 discloses an artificial beta cell produced by engineering endocrine cells of the At-T-20 ACTH secreting cells. A stably transfected cell At-T-20 ins is obtained by introducing cDNA encoding human insulin and the glucose transporter gene GLUT-2 driven by the constitutive CMV promoter. The cell line already expresses the correct isoform of glucokinase required for glucose responsive expression of the insulin gene. This cell line is responsive to glucose, but is regulated at a level of secretagogue below physiological range. Hence, while the system is of interest, it is not of clinical significance because an animal into which these cells are introduced would be chronically hypoglycemic. Another disadvantage is that the cells, being derived from a heterologous source, bear their distinctive foreign antigens, and must be used in immunoisolation. A further disadvantage is that At-T-20 is a transformed cell line with potential for unlimited growth.

U.S. Patent No. 5,534,404 discloses another approach to obtaining a correctly secretagogue regulated cell line. Starting with beta-TC-6 cells, subpopulations of cells are selected in an initial

stage by a cell sorter capable of recognizing cells having an increased internal concentration of calcium ion, associated with insulin expression (Ca++ activated fluorescence). After successive passages, cell populations are further selected which respond to glucose in the physiological 4 to 10mM range in a typical sigmoidal curve. For therapeutic use, the cells were encapsulated in alginate bounded by a PAN/PVC permselective hollow fiber membrane according to the method of Dionne (U.S. Patent application No. PCT/US92/03327).

Valera et al., FASEB Journal, 8: 440 (1994) describes transgenic mouse hepatocytes expressing insulin under control of the PEPCK promoter driven by P-enolpyruvate. The PEPCK promoter is sensitive to the glucagon/insulin ratio and is activated in elevated glucose states. The PECK/insulin chimeric gene was introduced into fertilized mouse eggs. Under conditions of severe islet suppression by streptozotocin, the production and secretion of intact insulin by the liver compensated for loss of islet function.

The strategy of gene therapy for treatment of diabetes is complicated by the complexity of insulin regulation and the structure of the protein itself. The glucose responsive release of insulin from the beta islet cells is a complex event involving migration of preprocessed protein from cytoplasm to the Golgi apparatus where secretory granules bud off and travel to and fuse with the plasma membrane prior to release. The initial protein product is preproinsulin having an N-terminal signal sequence, which is cleaved during transport to the rough endoplasmic reticulum. Thereafter the resulting proinsulin is further processed to insulin by removal of the C-peptide joining the two polypeptides of the mature molecule, the A and Ξ chains. In engineering the production of insulin in a host cell, it is impossible to obtain a

functional insulin by merely providing the A and B polypeptides. Synthesis of an intact molecule is necessary for proper folding, and only after the correct conformation is obtained, can the C-peptide be snipped out. Thus, any engineered cell expressing mature functional insulin must have the Kex2 enzyme machinery, including the PC1/PC3 and PC2 endopeptidases, or a functional substitute thereof, as suggested by Newgard, Biotechnology, 10: 1112 (1992).

The control of insulin production and release is further complicated by the regulation of glycolytic flux. It is believed that two proteins are used by the beta cell to sense changes in glucose levels: Glut-2 a specific facilitated diffusion type glucose transporter, and a particular glucose phosphorylating enzyme, glucokinase IV. Both enzymes have a higher Km and Vmax than the other enzymes in their related families. Both also have high affinities for glucose that result in large shifts in activity over the physiological range of glucose concentration. While reduction in GLUT-2 results in depression in insulin production, loss of glucokinase abruptly halts insulin production, and identifies glucose phosphylation as the true rate limiting step. Transformation of cells with expressible genes for these enzymes appears to restore glucose responsive regulatory characteristics to insulin production, but not infrequently outside the physiological range of control. The experiences many researchers have had underscores the problems inherent in the complexity of the control of insulin production through manipulation of the metabolic utilization of glucose. There is thus a need in the field of diabetes for a new model of insulin regulation and beta cell replacement.

SUMMARY OF THE INVENTION

Control of insulin production in synthetic beta cells may be accomplished by alternative regulatory pathways than through attempted restoration of natural control over a transformed beta cell expression system. While the actual release of insulin in normal beta cells is modulated through metabolic intermediates, as yet poorly understood, an alternative control is at the level of transcription of the mRNA encoding the proinsulin precursor. It is thus an object of the present invention to provide a control system for expression of the proinsulin gene in a suitable host cell, which is independent of the metabolic effectors and intermediates involved in normal regulation.

It is a further object to provide a replacement beta cell autologous with the patient's own cells to avoid a requirement for immunoisolation of insulin-producing cells. Ideally, a cell population is to be selected which can be engineered to synthesize insulin dependent on regulated gene transcription, without excision and extracorporeal manipulation outside the body. It is a further object to provide a gene therapy utilizing a cell population having intact and normally functioning glucose transporter and phosphorylating system, so that control of insulin production is a function only of transcriptional control. Consistent with this object is provision of an enzyme system capable of generating active insulin or insulin-like analog from proinsulin not subject to feedback intervention of the glycolytic pathway.

In accordance with the present invention, a gene cassette for expression of proinsulin in autologous host cells comprises a nucleotide sequence coding substantially full length proinsulin DNA, most preferably insulin cDNA, operably linked to a promoter recognized by an RNA polymerase contained in the host cells, together with a glucose responsive regulatory module having at least two glucose inducible regulatory elements located upstream at the 5' end of the

promoter. The cassette is integrated into a vector comprising a replication defective viral genome capable, when infecting a suitable target cell in vitro, of packaging the vector in a viral particle infective for the autologous host cells. The preferred target host cell is the hepatocyte because liver cells already express GLUT-2 and glucokinase IV sufficiently to generate the appropriate intermediates for glucose regulated transcriptional control of the proinsulin gene in the physiological range.

Hepatocytes also express the endopeptidase furin. A mutation can be introduced into the reading frame of the proinsulin gene that permits furin cleaving at the appropriate site to obtain substantially complete excision of the C-peptide with appearance of essentially native insulin activity. It is therefore an aspect of the present invention that in the transfection method, a vector is provided in which transcriptionally controlled production of proinsulin is substantially completely converted to the active hormone, which is constitutively secreted into the liver parenchyma in response to elevation in glucose concentration.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic depicting the nucleotide sequence of the glucose regulatory modules C and F respectively.

Figure 2 is a genetic map of the pACCMV.plpA 8.8 kb plasmid containing the cloning sites for the expression cassette for proinsulin, and also several adenoviral genes.

Figure 3a is a genetic map showing the insertion diagram of the expression cassette in relation to

various markers on the pACCMV.p.A plasmid. Figure 3b shows the order of genetic elements 5' to 3'.

Figure 4 is a genetic map of the large 40.3 kb pJM17 plasmid used to create the final recombinant vector for transfection.

Figure 5 is a genetic map showing the recombination of vectors pACCMV.plpA and pJM17 to yield the AdC/FAM construct.

Figure 6 is a gel reproduction of a Northern blot of RNA isolated from hepatocytes transfected with the recombinant plasmid vector containing the expression cassette, and cultured in the presence of various levels of glucose.

Figure 7 is a gel reproduction of a Northern blot identical to figure 6, only showing the result of a longer exposure of the CMV control.

Figure 8 is a duplicate experiment of that depicted in Figure 6 only showing the migration position of a control band of mRNA under control of the constitutive CMV promoter.

Figure 9 is a gel reproduction comparing rRNA bonding with mRNA from hepatocytes in the presence of various levels of glucose.

Figure 10 is a schematic diagram of the steps used to clone M2A and M2B.

Figure 11 is a graph comparing the amount of insulin released from Cos7 cells transfected with recombinant plasmids.

Figure 12 is a graph comparing the secretion and intracellular retention of insulin in Cos7 cells transfected with recombinant plasmids.

Figure 13 is a autoradiograph demonstrating the effect of time on glucose inducible upregulation of hIns mRNA in transfected hepatocytes. (Lanes 1,2, and 3- hIns mRNA expression at 27.5 mM glucose for .5h, 1h, and 16h, respectively. Lanes 4, 5, and 6-hIns mRNA expression at 5.6 mM glucose for .5h, 1h and 16h, respectively.)

Figure 14 is an autoradiograph demonstrating that glucose responsive human insulin production is observed in cell lysates from transduced rat hepatocytes. (Cells were transfected with the glucose inducible construct (Lanes 1 and 2) and cultured in 3.3 mM glucose (Lane 1) or 27.5 mM glucose (Lane 2). Lane 3 is a control lane of cells transfected with proinsulin under the control of a CMV promoter. Lane 4 represents the same material as in lane 3, except that an excess of unlabelled insulin was added prior to the immunoprecipitation step with insulin specific antibody.)

Figure 15 is a bar graph comparing insulin produced by hepatocytes transfected with a construct containing 1 GIRE at different glucose concentrations.

Figure 16 is a bar graph comparing insulin produced by hepatocytes transfected with a construct containing 2 GIREs at different glucose concentrations.

Figure 17 is a bar graph comparing insulin produced by hepatocytes transfected with a construct containing 3 GIREs at different glucose concentrations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In the present invention, replacement beta cells for treatment of diabetes Type I are constructed by transfection of autologous cells, preferably hepatocytes, with a vector expressing proinsulin genetically modified to be cleavable to insulin by an enzyme or enzymes endogenous to the transfected cells. Initially, a gene cassette is constructed containing the proinsulin gene and control elements suitable for its expression regulated by a secretagogue, preferably glucose.

In the isolation of native proinsulin cDNA, total RNA from normal human islet cells was extracted, and the mRNA fraction was isolated and used as a template in an oligo $(dT)_{15}$ primed reverse transcription reaction. Insulin cDNA (-28bp-443bp) was amplified using sense and antisense oligonucleotides which included restriction sites for KpnI and SalI, respectively. The sequences are shown in Table 1 designated TA423 and TA413, and are listed herein as SEQ ID NOS: 1 and 2. Alternatively, cDNA can be isolated according to the methods described in Bell, et al., Nature, 282: 525 (1979) using the primers disclosed therein, but incorporating restriction sites compatible with the selected cloning vehicle. In general, it is desirable to include in the amplified region a portion of the intron flanking the open reading frame of the proinsulin gene. The amplified DNA fragment (-28-443bp) containing the entire coding sequence of human insulin and a portion of the untranslated region from the 5' and 3' ends was subcloned into pBlueScript SK+. pBlueScript SK+ is a 2.96 kb colony-producing phagemid derived by replacing pUC19 polylinker of pBS(+/-) with a synthetic polylinker. This cloning vehicle is described in Shon, et al., Nuc. Acids Res., 16: 7583 (1988).

In a preferred embodiment of the invention, hepatocytes are transfected with a vector having a glucose regulated proinsulin gene. Hepatocytes express

an endogenous endopeptidase furin. Although furin is known to cleave proinsulin at its B-C junction, it is very inefficient at cleaving the C-A junction. Cleavage at both sites is required for excision of the C-peptide required for conversion of proinsulin to active insulin. A single point mutation (T^{267} to G converts the amino acid sequence KQKR to RQKR producing a modified C-A junction compatible with the specificity of furin. Thus, the proinsulin protein can be processed to insulin utilizing a single endogenous enzyme.

The mutation creating the new C-A junction may be effected by standard methods known in the art. For example, conversion of Lys to Arg can be made in two steps. The sense oligonucleotide (TA403 designates SEQ ID NO: 3) including a point mutation corresponding to the desired change in the target region was used with the original insulin antisense oligonucleotide (TA413) to amplify one segment of insulin. Similarly an antisense oligonucleotide (TA404) containing the Lys to Arg mutation was used with the original insulin sense oligonucleotide (TA423) to amplify the second fragment of modified insulin (M1). The two fragments, thus produced, can be purified, and a mixture of them used as template DNA in amplification of C-A modified insulin M1 with oligonucleotide TA423 and TA413. The C-A modified insulin M1 may be subcloned in pBlueScript SK+ at the restriction sites Kpn I and Sal I. In practice, the InsM1 DNA was sequenced and found to be error free. TA413, TA404, and TA423 are shown in Table 1, and are listed herein as SEQ ID NOS: 2, 4 and 1.

The key aspect of the invention is the control elements which make the transcription of the proinsulin gene responsive to the levels of extracellular glucose. Since the enzymes GLUT-2 and glucokinase are believed essential for glucose "sensing", hepatocytes, which produce the enzymes, make a good candidate for a replacement beta cell. Although, the mechanism of

"sensing" is not known, Applicants postulated that in addition to GLUT-2 and glucokinase, the rest of the "sensing" machinery would be intact in hepatocytes, including formation of any substances mediating gene expression at the transcriptional level.

To test this hypothesis, Applicants utilized varying combinations of different glucose inducible regulatory elements to determine the time course of insulin mRNA upregulation. As used herein, a glucose inducible regulatory element (GIRE) contains two perfect CACGTG motifs separated by five base pairs.

In the first set of experiments, the first module utilized, designated sequence C, contained four perfect CACGTG motifs. The second module, designated sequence F (SEQ ID NO: 5), was based on the module found in fatty acid synthetase. The first 21 bp constitute a perfect match of glucose inducible module containing two perfect CACGTG motifs but then an 11 bp long segment (10-20 bp of the oligonucleotide), CACGTGGGCGC, is repeated a plurality of times (at least twice, and preferably three to six times), creating a series of glucose inducible regulatory elements joined head to tail.

These constructs were inserted upstream of the 5' untranslated region of human preproinsulin gene and then cloned into an adenovirus vector which was used to transfect hepatocytes. Two sets of experiments were conducted. First, the sensitivity of regulation in response to physiological levels of glucose was analyzed by Northern blotting. Strong induction of insulin mRNA synthesis seen at glucose concentrations of greater than 5.5 mM. The gels show that both glucose regulatory modules, as described, are functional to about the same degree, although the AdFAM construct using the F module appears to be somewhat more responsive. Next, the temporal response of the construct containing module C to glucose was tested. This experiment demonstrated that upregulation of

insulin mRNA occurs within 30 minutes of exposure to supraphysiological levels of glucose. Together, these experiments demonstrate that GIREs provide transcriptional regulation of insulin mRNA synthesis in hepatocytes. Furthermore, the constructs respond within a time period necessary for use in treating diabetes, in contrast to Shih et al., J. Biol. Chem., 269: 9380 (1994, which measures total accumulation of CAT at 48 hours.

Further analysis of the construct described above indicated that the rat albumin promoter and insulin 5'-untranslated region contained an approximately six base pair inverted repeat. This inverted repeat has the potential to form a loop in the insulin mRNA, which inhibits post-transcriptional processing and translation.

To address this the problem, two chimeric gene cassettes including the 5'-untranslated region of rat albumin (bases 153-188 of the sequence published as GenBank Accession No. M16825, incorporated herein by reference) fused to the cDNA for human preproinsulin were amplified by PCR as described in Example 3. The insulin cDNA was previously modified to be cleavable by furin as described above. The constructs were designated M2A and M2B. These constructs differed only in that M2A terminated at the end of the human preproinsulin cDNA at base 382 while M2B includes 18 bases of the 3'-untranslated region of human preproinsulin. These promoterless constructs were then cloned into plasmid pcDNA3, which contains a CMV promoter. Expression from these plasmids was assayed in Cos7 cells. The data (presented in Fig. 11 and 12) demonstrates that these constructs are capable causing the production and secretion of insulin.

Applicants also compared insulin production from constructs containing either 1 (SEQ ID NO: 8), 2 (SEQ ID NO: 9) or three (SEQ ID NO: 10) GIREs incombination with the rat albumin promoter, rat albumin 5'

untranslated region and preproinsulin sequence M2B. The complete sequences of these constructs, which were inserted into adenovirus vectors, are listed as SEQ ID NO: 11 (containing 1 GIRE), SEQ ID NO: 12 (containing 2 GIREs), and SEQ ID NO: 13 (containing 3 GIREs). Insulin production in hepatocytes transfected with these vectors was analyzed. Hepatocytes transfected with the vector containing 1 GIRE demonstrated only low levels of insulin production. Hepatocytes transfected with the vectors containing either 2 or 3 GIREs demonstrated high levels of insulin production in response to physiologically relevant levels of hyperglycemia. It is expected that the baseline production of insulin will plateau with increasing numbers of GIREs. Furthermore, the construct containing 3 GIREs was more responsive to 10mM glucose than the construct containing 2 GIREs.

Shih, et al., supra, discloses different combinations of perfect and imperfect motifs in combination with a L-PK TATA box, driving a CAT gene. The first construct consists of the wild type L-PK promoter driving CAT. The GIRE in this promoter consists of two imperfect motifs, having the sequences CACGGG and CCCGTG. Each of these motifs contains a one base pair mismatch. The second construct consists of a S14 wild type GIRE in combination with the L-PK TATA box, driving a CAT gene. The GIRE in this construct consists of one perfect motif with the sequence CACGTG and one imperfect motif, having a two base pair mismatch, with the sequence CACGCG. The third construct consists of two S14 wild type GIREs, combined with the L-PK TATA box, driving a CAT gene. This construct has two GIREs, each having a perfect CACGTG motif and an imperfect motif, having a two base pair mismatch, with the sequence CACGCG.

CAT expression from these constructs was measured after the cells were cultured in 5.5 or 27.5 mM glucose for 48 hours. CAT activity in cell extracts

was expressed as a percentage conversion of chloramphenicol to its acetylated form. The L-PK construct demonstrated high expression of CAT after 48 hours in 27.5 mM glucose. Note that the motifs in this construct contained a one base pair mismatch. The construct containing one S14 wild type GIRE consisting of a perfect CACGTG motif and an imperfect motif with a two base pair mismatch displayed only low levels of CAT expression. The vector consisting of two S14 wild type GIREs, the GIREs consisting of one perfect motif with the sequence of CACGTG and one motif with a two base pair mismatch, also exhibited high levels of CAT expression.

The reporter system used for assaying glucose inducible regulation of transcription by GIREs in *Shih et al.* measured CAT activity after cells transfected with the vectors had been cultured with glucose at concentrations of 5.5 or 27.5 mM for 48 hours. Therefore, this gene expression assay measured the accumulation of the CAT protein in cell extracts, and did not measure mRNA synthesis directly. Therefore, the accumulation of CAT activity in the cell extracts is consistent with the slow up-regulation of transcription from the CAT gene. The Applicants' data demonstrates that constructs containing two GIREs expressed high levels of insulin mRNA after only 30 minutes of exposure to glucose.

The regulatory module for transcriptional responsiveness to glucose in the present invention is a synthetic oligonucleotide having at least two GIREs, each GIRE containing the two operative regulatory motif segments CACGTG flanking a nucleotide linker segment, conveniently of the sequence GGCGC. Preferably, the regulatory module contains from 2 to 8 GIREs, and most preferably the module contains from 3 to 5 GIREs. Additional GIREs are not believed to effect futher upregulation as a plateau of biding efficiency is to be expected. However, additional GIREs above 5 are not

expected to be detrimental to regulating function. The ends of the double stranded oligonucleotide module are synthesized to include half site restriction sequences to facilitate cloning. For example, for cloning in the preferred defective viral vector described hereinafter, each sense oligonucleotide starts with a Not I half site on the 5' end, and each antisense oligonucleotide includes an Eco RI half site on the 5' end.

A functional cassette includes the proinsulin gene, the glucose regulatory module, and a promoter. The promoter is preferably a relatively strong constitutive promoter normally operative only in the host cell of choice, and responsive to the regulatory module located on its 5' end. In the cassette devised herein, the rat albumin promoter happened to be selected, although many other candidates are known in the art. Using the published sequence, (Heard, et. al., "Determinants of Rat Albumin Promoter Tissue Specificity Analyzed by an Improved Transient Expression System", Mol. Cell. Biol., 7: 2425-2434 (1987), incorporated herein by reference) PCR primers were synthesized containing Eco RI and Kpn I restriction sites, as indicated in Table 1 and designated SEQ ID NOS: TA420 (6) and TA421 (7). Nucleotides 1-184 were thereupon amplified. The amplified rat albumin promoter fragment was purified, and cut with restriction enzymes Kpn I and Eco RI. After cloning into pBlueScript, the sequence was verified by conventional sequencing techniques. Preferably the PCR amplification is carried out utilizing the pfu polymerase obtainable from Stratagene, which has a significantly lower error rate than other polymerases.

The 5' untranslated region of insulin is replaced with the 5'-untranslated region of rat albumin fused in correct reading frame sequence (in native orientation) with substantially full-length human preproinsulin cDNA. Those skilled in the art will understand that

other 5'-untranslated regions may be substituted for the rat albumin 5'-untranslated region. It is important that adequate spacing (about 25 base pairs) be provided between the GIRE module and the transcription start site and that the 5' untranslated region contains as little secondary structure as possible so that there is proper binding to and processing by ribosomes. The use of the 5'-untranslated region of rat albumin may therefore be seen as a guide to the construction of other cassettes. Substantially full-length human preproinsulin cDNA means the full-length human preproinsulin cDNA as well as other truncated or substituted preproinsulin cDNAs and genomic DNAs, the transcription and translation of which produce insulin having biological activity.

The cassette comprising, 5' to 3', a glucose regulatory response module, a transcriptional promoter whose level of transcription can be further increased by the glucose response module, and the structural gene for proinsulin genetically modified to be cleavable by a host cell endogenous endopeptidase is spliced together and ligated by conventional techniques. The molecular ends of the polynucleotide cassette preferably have single stranded sequences defining the half restriction site corresponding to complementary half sites on the vector into which it is to be inserted.

The best available vector is a helper-free replication defective plasmid derived from the adenovirus genome, and described in Newgard, et al., "Glucose-Regulated Insulin Secretion," in Molecular Biology of Diabetes, eds. Draznin, et al., Humana Press: 1992. Figures 2-5 diagram the genetic components and construction of the vector containing the gene cassette. The advantages of this vector include the absence of helper virus, thus preventing propagation of virus and high efficiency of infectivity of host cells. It has the disadvantage of being

diluted out of replicating cells, since adenovirus integrates the host cell genome with very low efficiency. Other transducing systems useful in the present invention include certain integrating retroviral systems and another helper-free recombinant adenoviral system disclosed in U.S. Patent No. 5,436,146.

Another advantage of the viral-derived vectors is that delivery to target cells in the intact animal does not require excision of tissue, invitro infection, and reimplantation. Nothing however, would preclude the use of an allogenic source of cells under conditions of immunosuppression. A purified viral stock (2-40 infective units per target cell) may be injected into the hepatic portal vein, with efficient infective rates obtainable as viral particles penetrate the hepatic capillary beds and come into contact with the hepatocytes. In this way, replacement beta sites are generated insitu without disturbing the normal cellular architecture. Further advantages of the present invention will become apparent from the Examples which follow. Alternatively, hepatocytes may be transfected ex vivo and then transplanted into the human.

It will be apparent that any structural gene for which glucose modulated control is desired, may be inserted into the gene cassette by conventional recombinant techniques and expressed in an appropriate host cell. For proteins not requiring further processing, the fucin enzyme of hepatocytes is, of course, superfluous. A number of metabolic diseases for which the present invention has therapeutic value in restoring a glucose response mediated protein function can be identified.

EXAMPLE 1

<u>Generation and Cloning of Insulin Gene Cassettes in the plasmid pACdeltaCMV.</u>

The plasmid pACCMV.pLpA (Fig. 2), used as a vector for generation of replication defective recombinant adenovirus containing genes of interest, was cut to completion with the restriction enzyme Sal I and partially with the enzyme Not I. The 8.3 kb piece of KNA, lacking CMV promoter, was gel purified and used as vector for inserting insulin gene cassettes.

The oligonucleotide pair corresponding to one of the GIREs was mixed with gel purified Eco RI - Kpn I albumin promoter and Kpn I - Sal I InsM1 DNA fragments, the mixture was ligated with the above described plasmid vector pACΔCMV. A combination of a Glucose Regulatory Response Modules C or F (Figure 1), albumin promoter and the mutant insulin cDNA, produced a total of two constructs. Integrity of both the constructs was confirmed by sequence analysis. Each of the two constructs was cotransfected with the plasmid pJM17 in the host 293 cell line, as described, to generate recombinant replication-defective adenovirus constructs, namely Ad.CAM1 and Ad.FAM1 (see Figure 5).

EXAMPLE 2

Expression of Insulin in Hepatocytes at various glucose concentrations.

Rat hepatocytes were prepared by *in situ* perfusion of 0.5 mg/ml collagenase in supplemented balanced Hank's solution as described (Kreamer et. al. (1986) In Vitro 22, 201-211). The viability of isolated hepatocytes was 90% or better.

Six collagen coated 60 mm plates, each containing $1x10^6$ hepatocytes, were transfected with $5x10^7$ pfu/plate. The transfected hepatocytes were exposed to three concentrations of glucose, 3.3 mM, 5.6 mM and 27.5 mM, in RPMI supplemented with 10% fetal calf serum, 30 μg/ml proline, 5 μg/ml insulin, 5 μg/ml transferrin and 5 μg/ml selenium. After 36 h, one of the two plates at each of the tested glucose

concentrations, was used to prepare RNA and the other plated was used to check the viability of the hepatocytes. Hepatocyte viability at all the tested concentrations of glucose were no more than 10% different. Ten microgram of RNA from each sample was electrophoretically resolved on a formaldehyde-2% agarose gel, the RNA transferred to a Nylon membrane, UV-crosslinked and hybridized with digoxygenin-labeled insulin cRNA. Detection of the membrane-bound probe was performed by chemiluminescence, results recorded as multiple exposures on X-ray films for various lengths of time and quantitated by digital image analysis.

Referring to figures 6 and 7, Northern analysis reveals that RNA migrating at the position of polynucleotides of approximately 1.35 kb, corresponding to the predicted size of the proinsulin transcript, is evident only when transfected hepatocytes are cultured in the presence of 27.5 mM glucose. Importantly, no induction of the proinsulin gene over background is indicated at 3.3 or 5.5 mM glucose. Unlike other attempts at constructing an artificial inducible insulin-producing replacement cell, in which induction occurs at subphysiological levels of glucose, the present transfected hepatocytes show a response only in physiological or supraphysiological range. Strong induction is seen at glucose concentrations of greater than 5.5 mM. A strong response is apparent at 10mM. The gels show that both glucose regulatory modules, as described, are functional to about the same degree, although the AdFAM construct using the F module appears to be somewhat more responsive. As a control, the Ad.CMP-Ins in which the gene of interest (proinsulin or beta-galactosidase) is under control of the constitutive CMV promoter, generates RNA of the distinctive size without regard to glucose concentration and is used to quantitate the amount of insulin mRNA (Figures 6, 7 and 8).

Quantitation by phosphoimaging is summarized in Table 2. The results show only a slight difference in relative expression between 3.3 and 5.6 mM glucose, in contrast to a 3.06 value at 27.5 mM. Figure 9 shows the gel normalizing rRNA and mRNA to arrive at the 3.06 value.

Table 1

Oligo	Sequence (5'-3')
TA403	GAG GGG TCC CGG CAG AAG CGT GGC A
TA404	ACG CTT CTG CCG GGA CCC CTC CAG G
TA413	Sal 1 CGG AGT CGA CCA TCT CTC TCG GTG CAG GAG GCG G
TA420	Eco R1 GGA ATT CTC TAG AGG GAT TTA GTT AAA CAA CTT
TA421	Kpn 1 GGG GTA CCA GAG GCA GTG GGT TGA CAG GT
TA423	Kpn 1 GGG GTA CCA TCA GAA GAG GCC ATC AAG CA

Table 2

Quantitation of Insulin mRNA in Hepatocytes

Glucose (mM)	hIns mRNA	18s RNA	Normalized hIns mRNA (hIns mRNA/18s RNA)	hIns mRNA (Relative* Expression)
3.3	81074	56	787	0.91
5.6	51039	33	865	1.00
27.5	201056	40	2645	3.06

*For the sake of comparison, the normalized amount of insulin mRNA expressed at euglycemic level (5.6 mM glucose) is arbitrarily assumed to be one.

EXAMPLE 3:

CLONING OF INSULIN CONSTRUCT WITH RAT ALBUMIN 5' UNTRANSLATED REGION

The chimeric insulin genes of Example 1 were further modified by eliminating the 5' untranslated region of human insulin mRNA and replacing that region with the 5' untranslated region of rat albumin (bases 153-188 as published in GenBank Accession No. M16825, herein incorporated by reference). This modification was accomplished by PCR amplification of hInsM2 using three oligonucleotide primers (sequences listed in Table 3). The oligonucleotide TA455 (5'-3') comprises a sequence corresponding to the restriction enzyme Kpn I recognition site, bases 153-188 of the albumin promoter corresponding to the 5'-untranslated region of albumin and a sequence corresponding to human preproinsulin mRNA (starting with the first amino

acid). The reverse (antisense) oligonucleotide TA452 terminates at the end (base 392) of the translated sequence of the human insulin cDNA sequence. The reverse (antisense) oligonucleotide TA454 corresponds to the 3' region of human preproinsulin CDNA plus 18 bases of the 3' untranslated region of human insulin (bases 393-410). Both of these reverse oligonucleotides also included a Sal I restriction site to facilitate cloning of the generated products into the small pACCMV based plasma described in Example 1. The amplification product of TA455 and TA452 has been designated as mutant M2A. The amplification product of TA455 and TA454 has been designated as the mutant M2B.

After amplification, the modified chimeric insulin genes were then cloned without their promoter sequences into the commercial plasmids vector pcDNA3 containing the CMV promoter. The steps involved in cloning and expression are schematically depicted in Figure 10. Since pcDNA3 does not have a unique Sal I site, the orientation of insertion was controlled by using Kpn I and EcoRV. The insulin gene mutants M2A and M2B were digested with Kpn I and separately ligated into the pcDNA3 vector. After transformation of E. coli using the ligated DNA, plasmids containing the two insulin mutants were prepared and used to transfect Cos7 cells.

EXAMPLE 4:

SECRETION OF INSULIN FROM COS 7 CELLS TRANSFECTED WITH INSULIN CONSTRUCTS CONTAINING THE 5'-UNTRANSLATED REGION OF RAT ALBUMIN

Cos7 cells were used to test the ability of the constructs described in Example 3 to synthesize and secrete insulin. The TRANS IT™ transfection reagent (Pan Vera Corp., Madison, Wisconsin) was used to transfect the Cos7 cells with plasmids containing the M2A and M2B mutants for transient expression. A four-

hour period of incubation for transfection was followed by an overnight incubation of the cells and fresh DMEM supplemented with 10% fetal calf serum. The medium was then changed and the plates incubated for two days in medium containing either 10% or 5% fetal calf serum. The medium and the cells were harvested separately, and the cells lysed in Tris-buffered saline (pH 7.6) containing 1% NP-40 and protease inhibitors (trypsin inhibitor and PMSF). Both the medium and cell lysate were analyzed for the presence of insulin by antigen capture ELISA.

The preliminary results demonstrate that both clones are capable of causing insulin synthesis and secretion. M2B demonstrates a tendency towards higher insulin production (see Figures 11 and 12). It is also apparent that the majority, if not all, of the synthesized insulin is secreted (Figure 12). Control cells transfected with the vector pcDNA 3 with no insert did not produce insulin. The measured levels of insulin in these controls was not significantly different that the baseline of the insulin standard curve.

TABLE 3

Oligonucleotides Used for Amplification of Insulin Mutants M2A and M2B

Sequence Name	Sequence Length	Sequence
TA455	64	*ggg gTA CCg* ACC ACC TTT CCT gTC AAC CCA CTg CCT CTg gCA CAA Tgg CCC TgT ggA TgC gCC T
TA452	30	*Cgg AgT CgA* CCT AgT TgC AgT AgT TCT CCA
TA454	30	*Cgg AgT CgA* CgC TgC CTg CAg gCT gCg TCT

Example 5:

Quantification of glucose-dependent insulin release from hepatocytes transfected with adenovirus constructs containing insulin and 1, 2 or 3 GIREs.

The examples above included data showing that glucose-inducible transcription of hIns in rat hepatocytes is achieved via a genetic construct with hIns cDNA under control of a chimeric serum albumin promoter containing glucose-inducible regulatory elements (GIREs). The information regarding the ability of hIns mRNA to produce and secrete insulin protein was also provided using COS7 cells.

To study expression in hepatocytes, three chimeric human insulin constructs in adenovirus, containing 1, 2 or 3 GIRE units were constructed. Followed by the specific number of GIRE(s), each construct contains albumin promoter and human insulin cDNA (including the two sets of mutations to aid furin mediated processing of proinsulin to insulin).

The general strategy of assembling the hIns constructs was essentially the same as described above. The sense and antisense oligonucleotides corresponding to 1 or 2 GIREs were chemically synthesized. Each set of oligonucleotide pairs was designed such that when annealed together, the double stranded DNA contained the sticky ends for the restriction enzymes Not I and EcoR I on 5′ and 3′-ends, respectively. An additional pair of oligonucleotides corresponding to one GIRE was made to contain EcoR I and XbaI sites on the 5′ and 3′-ends, respectively. The original sense oligonucleotide used for amplification of rat albumin promoter contains EcoR I site followed by Xba I site. The last pair of oligonucleotide (1GIRE) was inserted into the construct containing 2 GIREs using EcoR I-Xba I, thus giving rise to the construct with 3 GIREs.

The rat albumin promoter sequence described earlier was extended by PCR to include the entire 5′-untranslated region of rat albumin. The human insulin

cDNA containing two sets of mutations, corresponding to the B/C and C/A junction of proinsulin, from constructs previously described was modified to eliminate the 5'-untranslated region arising from hIns cDNA by PCR. The two fragments of DNA were joined together by overlap extension in a PCR reaction. The product of this reaction contained (5'->3') albumin promoter, 5'-UTR of albumin and translated sequence of hIns modified to be compatible with furin. This DNA fragment containing EcoR I and SalI restriction enzyme sites on 5' and 3'-ends, respectively, was digested with the two enzymes, mixed with the annealed pair of oligonucleotides corresponding to 1 or 2 GIREs, described above, and ligated into the plasmid vector used to produce adenovirus (pACCMV from which CMV promoter has been deleted).

Applicants next determined whether the glucose-induced upregulation of hIns mRNA is accompanied by a corresponding glucose-induced secretion of hIns protein and if the extent of glucose-induced insulin secretion varies depending on the number of GIRE units used per construct. Plasmids corresponding to 1, 2 or 3 GIREs were individually mixed with the plasmid pJM17 and the mixture of the two plasmid DNA cotransfected into HEK 293 cells to generate recombinant replication-defective adenovirus (as described earlier). Each recombinant hIns construct was sequenced to ascertain that no errors were introduced by PCR or during subsequent cloning procedures. Freshly prepared hepatocytes were plated in collagen coated 30mm plates and transfected (MOI=4) with adenovirus containing insulin construct with 1 (Ad1SAM2B), 2 (Ad2SAM2B) or 3 (Ad3SAM2B) GIREs, as indicated. The culture medium was changed 16h post-transfection, with medium containing 2.5, 5.6, 10 or 27.5 mM glucose, as indicated. The insulin present in medium was assayed by antigen capture ELISA 32h later (the numbers shown in Table 4 represent ng Insulin/ml +

SD). The results are presented in Figs. 15, 16 and 17 and in Table 4.

A glucose-dependent increase in the secreted insulin from transduced hepatocytes is clearly seen when the gene construct contains either 2 (Fig. 16) or 3 (Fig. 17) GIREs. However, in case of the construct having 1 (Fig. 15) GIRE, the overall insulin secretion as well as glucose induction is minimal. Furthermore, the construct containing 3 GIREs, in addition to a higher maximal induction at 27.5 mM glucose (approximately 9 fold increase over the level at 2.5 or 5.6 mM glucose as opposed to 6.5 fold increase in the case of construct with 2 GIREs under identical conditions), also shows >2 fold increase in insulin secretion at 10 mM glucose.

TABLE 4

Insulin secreted from Hepatocytes transfected with vectors containing 1, 2, or 3 GIREs

Glucose, mM	Ad1SAM2B	Ad2SAM2B	Ad3SAM2B
2.5	4.97 ± 1.09	9.09 ± 2.27	9.98 ± 1.37
5.6	5.12 ± 1.26	10.72 ± 2.30	10.80 ± 1.57
10.0	3.2 ± 1.8	13.2 ± 2.4	22.8 ± 4.1
27.5	12.75 ± 2.05	67.16 ± 10.24	90.33 ± 19.80

Example 6.

Temporal response of GIRE constructs to physiological levels of glucose.

Collagen coated 60 mm plates or dishes, each containing 1 X 10^6 hepatocytes, were transfected with 3.5 X 10^6 pfu/plate of the test adenovirus containing the insulin gene and two GIREs. Control plates received either no virus, or virus encoding bacterial B-galactosidase. Hepatocytes were then exposed for 16h

to 5.6 mM glucose in RMPI supplemented with 10% fetal calf serum, 30 μg/ml proline, 5 μg/ml insulin, 5 μg/ml transferrin and 5 μg/ml selenium, at 37 degrees Centigrade. The plates containing transfected cells were then divided into two groups, one group receiving fresh medium containing 5.6 mM glucose, the second group receiving fresh medium with 27.5 mM glucose. From each of these two groups, individual plates were removed after 30 min, 1h, 2h, 4h, 8h, and 16h, the medium decanted, and the cells frozen in liquid nitrogen. Total RNA was extracted and analyzed for hIns mRNA by Northern blotting.

The Northern blot in Figure 13 demonstrates that after exposure to 27.5 mM glucose, hIns mRNA was detectable at the first time point of 30 min and increased thereafter in a time-dependent manner. At the normal glucose level (5.6 mM) the signal was much lower. Quantitation of the bands revealed that the upregulation of insulin message observed at 27.5 mM is roughly 10 fold as compared to the 5.6 mM treatment.

These data demonstrate that the vector construct containing two GIREs initiates transcription in response to elevated glucose levels in a time-frame comparable to islet cells. This rapid temporal response to elevated glucose levels confers a level of control of insulin synthesis in addition to control mechanisms arising from glucose detection by the GLUT-2 and glucokinase pathways. These data also demonstrate a precise and correct response to physiological levels of glucose. Insulin mRNA production is not upregulated in the presence of 5.6 mM glucose, the steady state concentration of glucose in the bloodstream. Insulin mRNA synthesis is stimulated by elevated (above 5.6 mM) levels of glucose.

Example 7.

Insulin synthesis at physiological ranges by hepatocytes transfected with construct containing 2 GIREs.

Freshly prepared rat hepatocytes were transfected with two different adenovirus constructs containing the M1 mutated insulin gene: AdSAM1 (containing the rat Albumin promoter modified to contain 2 GIREs) and AdCMVInsM1 (containing the constitutive and highly active CMV promoter). Hepatocytes transfected with AdCMV.β-Gal and untransfected hepatocytes were used as controls. Four plates of hepatocytes were transfected with each adenovirus preparation; two plates were exposed to the low (3.3 mM) glucose and the other two plates to the high (27.5 mM) glucose concentration. After 36h, hepatocytes were exposed for 16h to 5.6 mM or 27.5 mM glucose in RMPI supplemented with 2 mg/ml bovine serum albumin with leucine omitted, 30 μg/ml proline, 5 μg/ml insulin, 5 μg/ml transferrin and 5 μg/ml selenium, at 37 degrees Centigrade.

Following a 6h leucine depletion, a 2 ml aliquot of the low or high glucose containing defined medium was added to appropriate plates. For each adenovirus used, one plate for each glucose concentration received 0.2 mCi ^{3}H-leucine (500 Ci/mmole). The remaining plate received the equivalent amount of unlabelled leucine and at the end of all incubations it was used for viability determination.

The leucine incorporation was carried out for 16h, followed by a 4h chase with unlabeled leucine. The culture medium was aspirated, cell debris removed, and the supernatant used for analysis of secreted products. The cells on each plate were lysed with 0.8 ml solution containing 20 mM Tris-HCL buffer at pH 7.6, 2 mM EDTA, 5 μg/ml trypsin inhibitor, 50 μM phenylmethane sulphonyl fluoride (PMSF) and 1% Triton-X100. The lysate was centrifuged at 16,000xg for 10 min in a

microcentrifuge, the pellet discarded, and the supernatant solution used for analysis of labeled intracellular products.

For each analysis of secreted insulin by immunoprecipitation, 0.8 ml of culture supernatant was used, and for each analysis of intracellular insulin 0.4 ml of cell lysate supernatant was used. Samples were pre-cleared with *Staph. aureus* in the absence of specific antibodies, to reduce the non-specific precipitation of labeled proteins during the procedure: 50μl of 10% suspension of formalin-fixed Staphylococcus cells (from Calbiochem) was added to each tube; tubes were kept at room temperature for 30 min with continuous mixing, centrifuged (4 min, 16,000xg) and the supernatant used for further analysis. For immunoprecipitation of insulin and insulin-related products, 2.5μl of polyclonal guinea pig anti-human insulin (from Sigma Chemical Co.) was added to the supernatant solution from the above step, mixed and kept at room temperature for 45 min, followed by addition of Staphylococcus cells with 30 min incubation plus centrifugation exactly as before. Supernatant was discarded, and the pellet washed 4-5 times with 1 ml of 20 mM Tris-HCl at pH 7.6, 0.15 M NaCl and 0.1% Triton X-100.

The pellet was suspended in 40 μl solution containing 60 mM Tris-HCl at pH 6.8, 1.2% SDS, 2% β-mercaptoethanol, heated in a boiling water bath for 4 min, centrifuged, and the supernatant analyzed by polyacrylamide-SDS gel electrophoresis. As an internal control for sample to sample variation, in a second set of tubes 2 μl of rabbit anti-rat albumin polyclonal antiserum was included along with anti-insulin antiserum, enabling co-precipitation of human insulin and the endogenous rat albumin in the subsequent single step. Specificity of the immunoprecipitated material was established by the use of control cells transduced with an unrelated gene, β-galactosidase, and

untransfected cells. To further confirm the identity of the immunoprecipitated material, in a separate set of tubes unlabeled insulin and rat serum were added to provide competition with labeled insulin and albumin respectively, and tubes processed simultaneously.

The optimum gel system for resolution of insulin B and A chains and rat albumin was found to be an SDS/Tris-Tricine 10-20% linear polyacrylamide gradient based on the description of Schagger and Jagow (Analyt. Biochem. 166:368-379, 1987). A 15 μl aliquot of SDS-βME-treated immunoprecipitated material from each sample was resolved on the gel along with peptide size markers from BioRad. The gels were fixed, stained, destained, soaked in "Amplify" solution (Amersham), dried under vacuum, and exposed to X-ray film at -80OC.

Our results (Figure 14) show the presence of an anti-insulin antibody-binding band in cell extracts of hepatocytes transduced with both of the insulin cDNA-containing constructs, AdSAM1 (glucose-inducible) and AdCMV.InsM1 (constitutive). As noted in Methods above, both constructs contain the M1 insulin cDNA, mutated in the coding region at the C/A junction. AdSAM1 contains two glucose-inducible regulatory elements coupled to the rat albumin promoter, and AdCMV.InsM1 contains the cytomegalovirus immediate/early promoter. When AdSAM1 was used, the insulin band appeared only in the hepatocytes exposed to high glucose and not the low glucose. When AdCMV.InsM1 was used, the insulin-positive band was present in approximately equal amounts regardless of the glucose concentration. It should be noted that these results were controlled for possible differences in gel loading and other sources of sample-to-sample variation, including a difference in hepatocyte survival after incubation at 3.3 mM versus 27.5 mM glucose (10-15% lower viability at low glucose), by co-precipitating rat serum albumin and using it as an internal standard.

The size of the insulin positive band was determined to be 7,700 Daltons. This differs from the sizes of mature insulin B and A chains and most likely contains C+B peptides of insulin as a result of incomplete processing proinsulin. The size of rat albumin was determined to be 67,000 Daltons, which compares favorably with the known size. The identities of the observed bands as insulin and albumin were confirmed by the fact that the signals were almost completely ablated when excess unlabeled insulin and normal rat serum were included during the immunoprecipitation. A preliminary determination using digital densitometry revealed that intra-cellular insulin protein expression at high glucose was only about 20-fold lower when driven by the chimeric albumin promoter employed in AdSAM1 then when driven by the CMV promoter in AdCMV.InsM1. This is highly encouraging since the CMV promoter is the most active known promoter in most *in vivo* and *ex vivo* mammalian systems. It would not be desirable to express insulin at the levels achieved by the CMV promoter.

These data demonstrate the synthesis of insulin in response to physiological levels of glucose in hepatocytes transfected with a vector containing the proinsulin gene under the control of two GIREs. At 3.3 mM glucose, there is no detectable sythesis of insulin; at 27.5 mM glucose, insulin synthesis is readily detectable. By adding two GIREs to the vector, which provide an additional control mechanism at the transcriptional level, the synthesis of insulin is correctly regulated in the appropriate physiological range. Insulin mRNA and insulin are synthesized at glucose concentrations exceeding 5.6 mM and are not synthesized at glucose concentrations of 5.6 mM. This feature makes vectors containing GIREs uniquely appropriate for treatment of Type 1 diabetes and superior to the prior art vectors.

TREATMENT OF DIABETES WITH SYNTHETIC BETA CELLS

CLAIMS

What is claimed is:

1. A gene cassette for expression of a proinsulin gene in a host cell comprising

a nucleotide sequence coding for a proinsulin cleavable into active insulin and operably linked to a promoter sequence recognized by an RNA polymerase contained in said host cell; and

a glucose responsive regulatory module located 5' of said promoter comprising four or more CACGTG motifs, said motifs separated by a linker sequence.

2. The gene cassette of claim 1 further comprising a nucleotide sequence coding for a 5' untranslated region of rat albumin located in native orientation between the promoter sequence and the proinsulin sequence.

3. A vector for transfecting a host cell capable of transcribing a proinsulin gene contained in said vector, said vector comprising

a gene cassette consisting of a nucleotide sequence encoding a proinsulin cleavable into active insulin and operably linked to a promoter transcribable in said host cell and a regulatory module located 5' of said promoter, and

a replication defective viral genome capable of expressing the genes required to package said vector invivo into a viral particle infective for said host cell.

4. The vector of claim 3 wherein said host cell is a hepatocyte.

5. A synthetic glucose responsive regulatory module comprising

four or more CACGTG motifs, said CACGTG motifs separated by a linker segment.

6. The glucose responsive regulatory module of claim 5 wherein said linker segment joining said CACGTG motifs is 5'-GGCGC-3'.

7. A gene cassette for glucose modulated expression of a structural gene in a host cell comprising

a nucleotide sequence coding for said structural gene operably linked to a promoter recognized by an RNA polymerase contained in said host cell; and

a glucose responsive regulatory module located 5' of said promoter comprising four or more CACGTG motifs, said motifs separated by linker sequence.

8. A method of treating diabetes comprising

transfecting cells expressing glucose transporter 2, glucokinase, and an enzyme capable of cleaving a genetically modified proinsulin to yield a protein having insulin activity, with a vector comprising

a gene cassette consisting of a nucleotide sequence encoding a substantially full length proinsulin gene operably linked to a promoter transcribable in said cells, and a regulatory module located 5' of said promoter,

a replication defective viral genome capable of expressing the genes required to package said vector invivo into a viral particle infective for said cells.

9. The method of claim 4 wherein said cells are hepatocytes.

Glucose Inducible Regulatory Elements

C

gC ggC CgC CAg TTC TCA CgT ggT ggC CAC gTg CTT ggg CAC
gCC AgTTCT CAC gTg gTg gCC ACg TgC TTg ggC ACg TTA AC

E

gC ggC CgC TgT CAC gTg ggC gCC ACg Tgg gCg CCA CgT ggg
CgCCAC gTg ggC gCC ACg Tgg gCg CCg TTA AC

FIG. 1

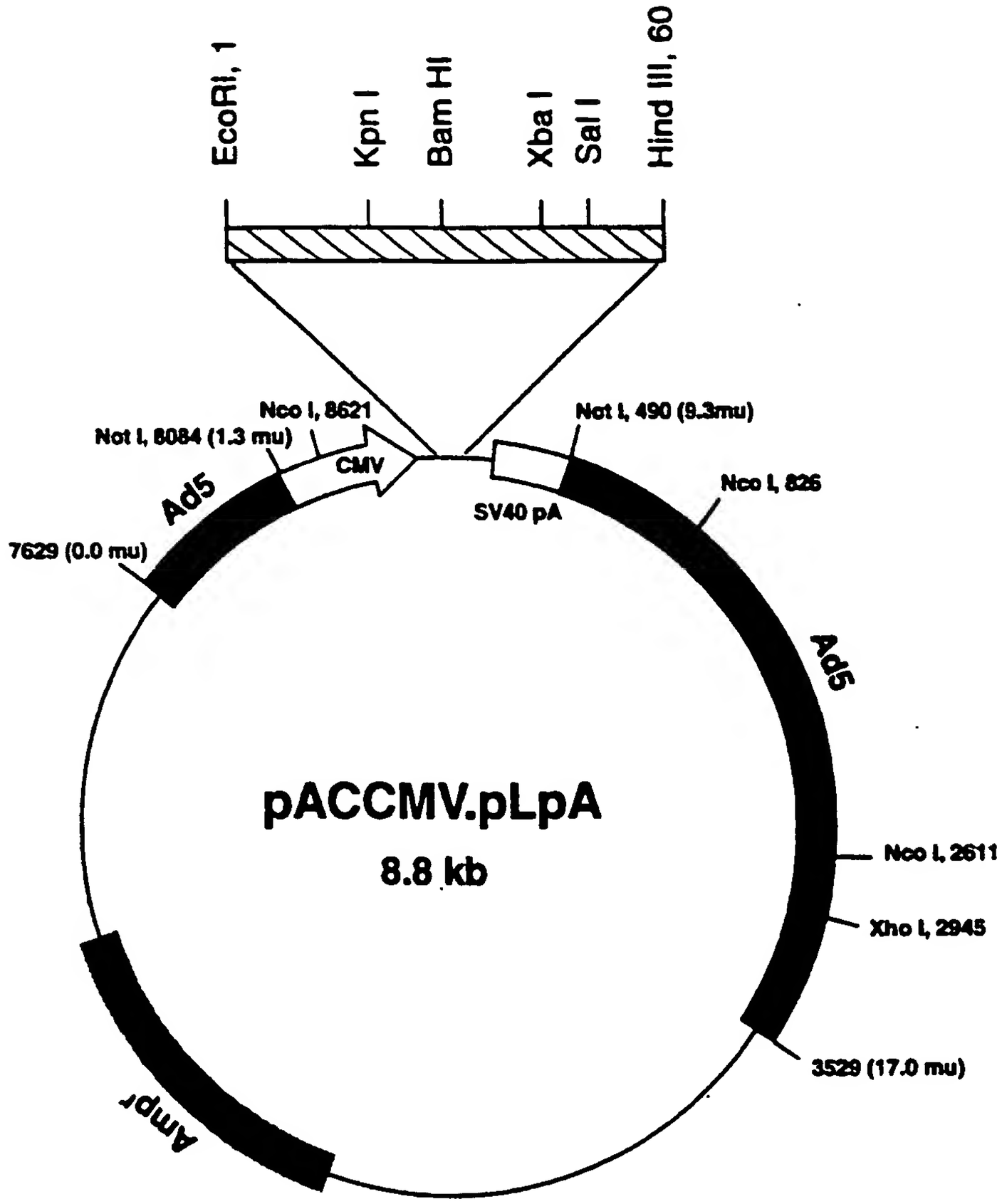
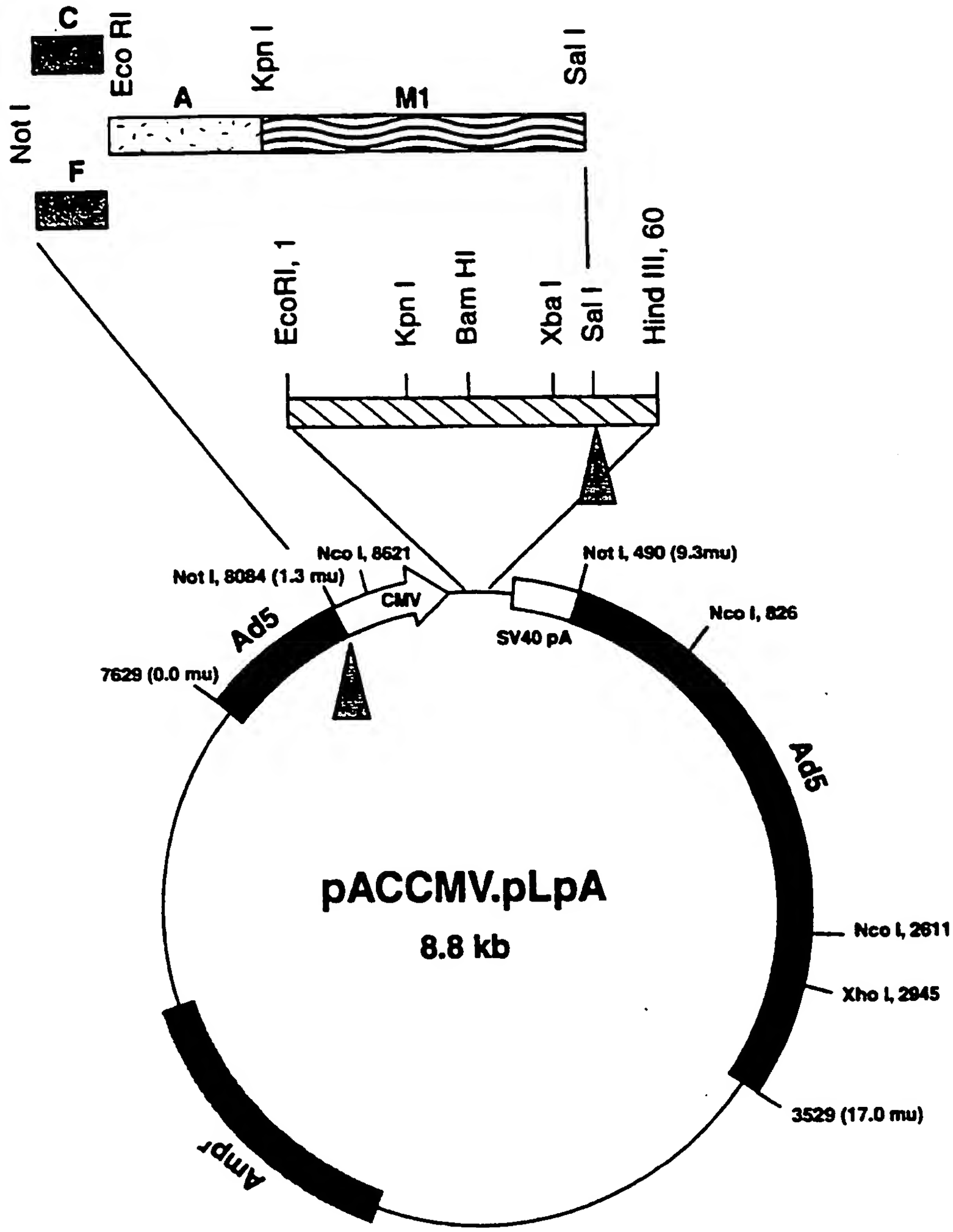

EcoRI, 1
Kpn I
Bam HI
Xba I
Sal I
Hind III, 60
Nco I, 8621
Not I, 8084 (1.3 mu)
CMV
Ad5
7629 (0.0 mu)
Not I, 490 (9.3mu)
SV40 pA
Nco I, 826
Ad5
Nco I, 2611
Xho I, 2945
3529 (17.0 mu)
AmpR
pACCMV.pLpA
8.8 kb

FIG. 2


C
Eco RI
Not I
A
Kpn I
M1
Sal I
F
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Kpn I
Bam HI
Xba I
Sal I
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Nco I, 8621
Not I, 8084 (1.3 mu)
CMV
Not I, 490 (9.3mu)
SV40 pA
Nco I, 826
Ad5
7629 (0.0 mu)
pACCMV.pLpA
8.8 kb
Ad5
Nco I, 2611
Xho I, 2945
3529 (17.0 mu)
AmpR

FIG. 3A

Insulin Expression Clones in Adenovirus

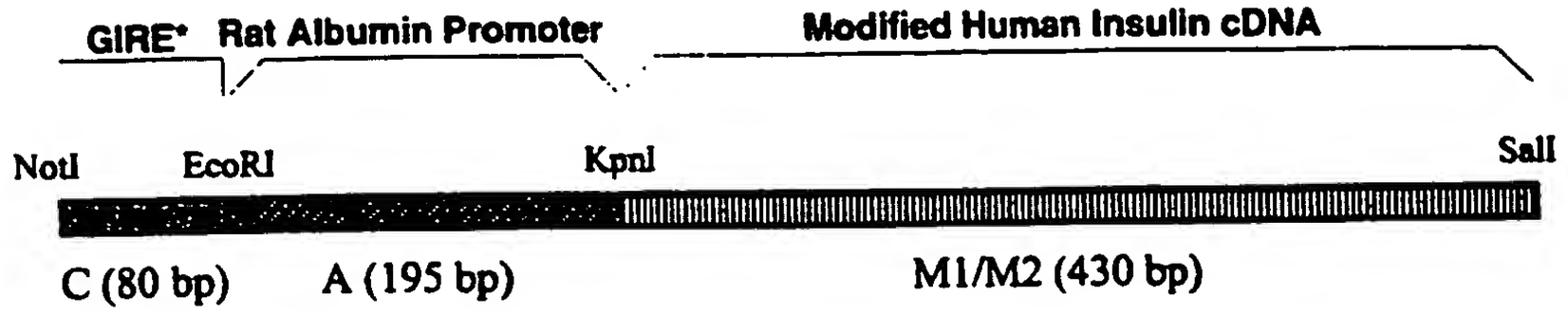

FIG. 3B

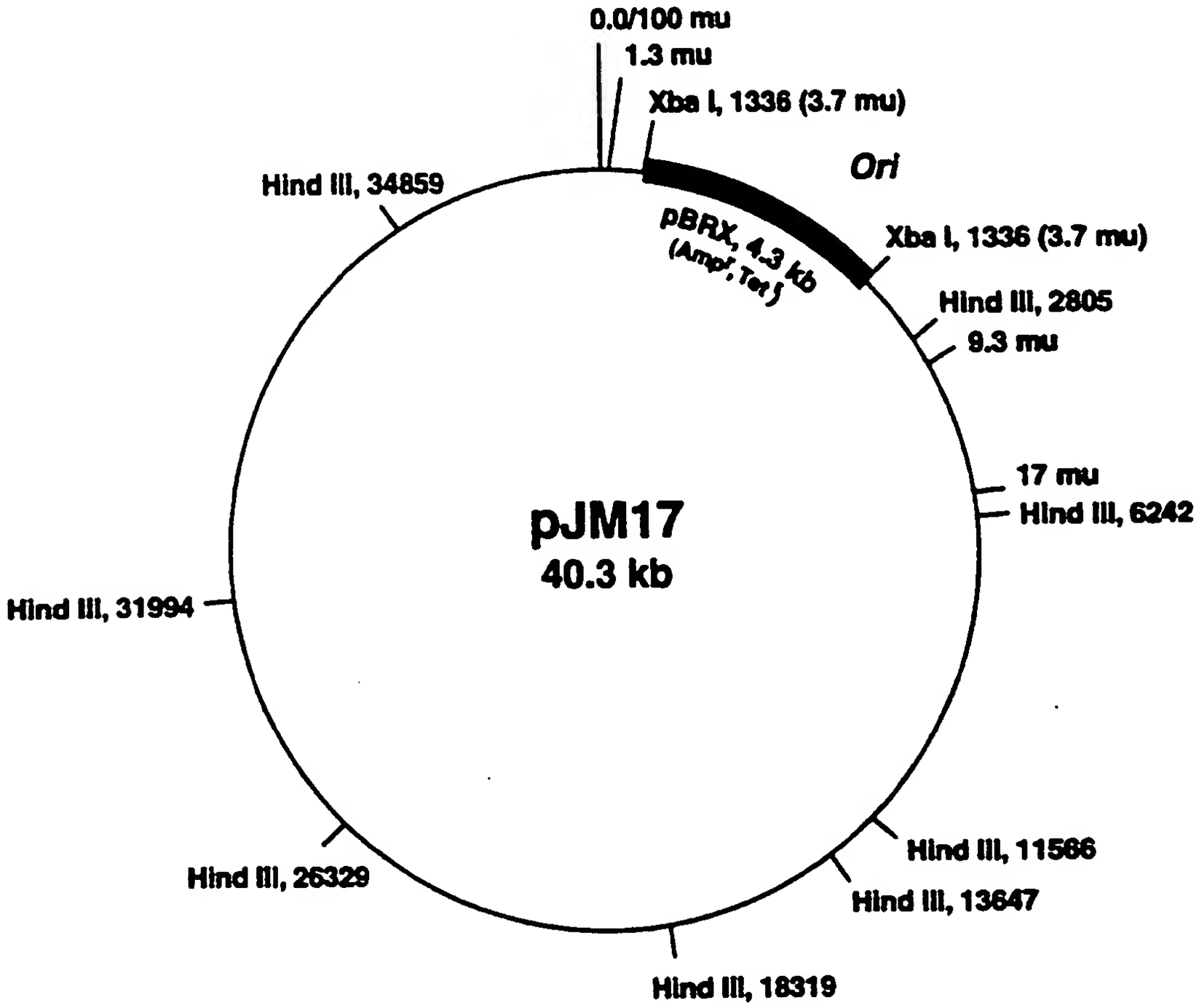
0.0/100 mu
1.3 mu
Xba I, 1336 (3.7 mu)
Ori
Hind III, 34859
pBRX, 4.3 kb
(Amp^r, Tet^r)
Xba I, 1336 (3.7 mu)
Hind III, 2805
9.3 mu
17 mu
Hind III, 6242
pJM17
40.3 kb
Hind III, 31994
Hind III, 26329
Hind III, 18319
Hind III, 13647
Hind III, 11566

FIG. 4

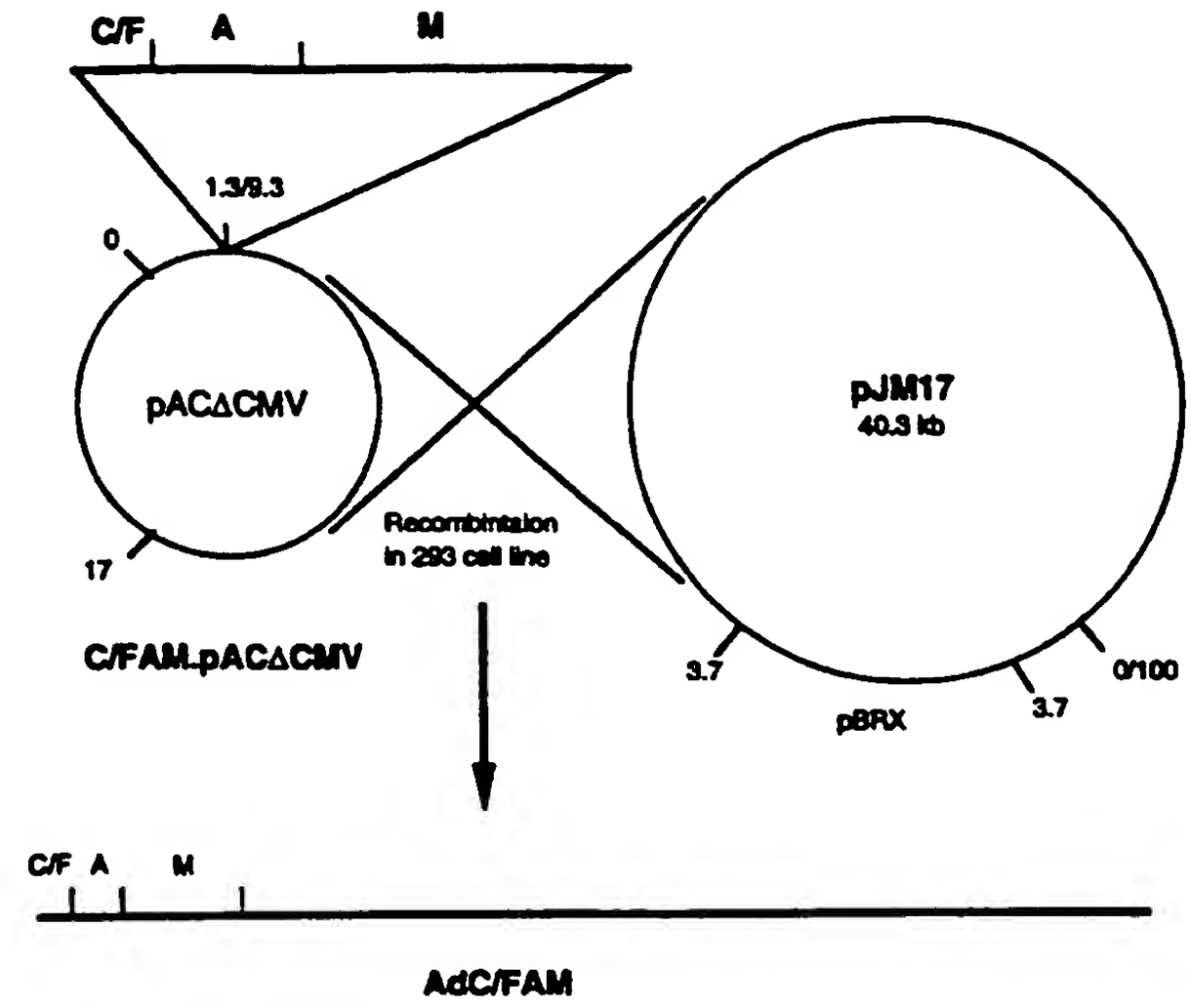
C/F
A
M
1.3/9.3
0
pACΔCMV
17
Recombination
in 293 cell line
C/FAM.pACΔCMV
pJM17
40.3 kb
3.7
pBRX
3.7
0/100
C/F
A
M
AdC/FAM

FIG. 5

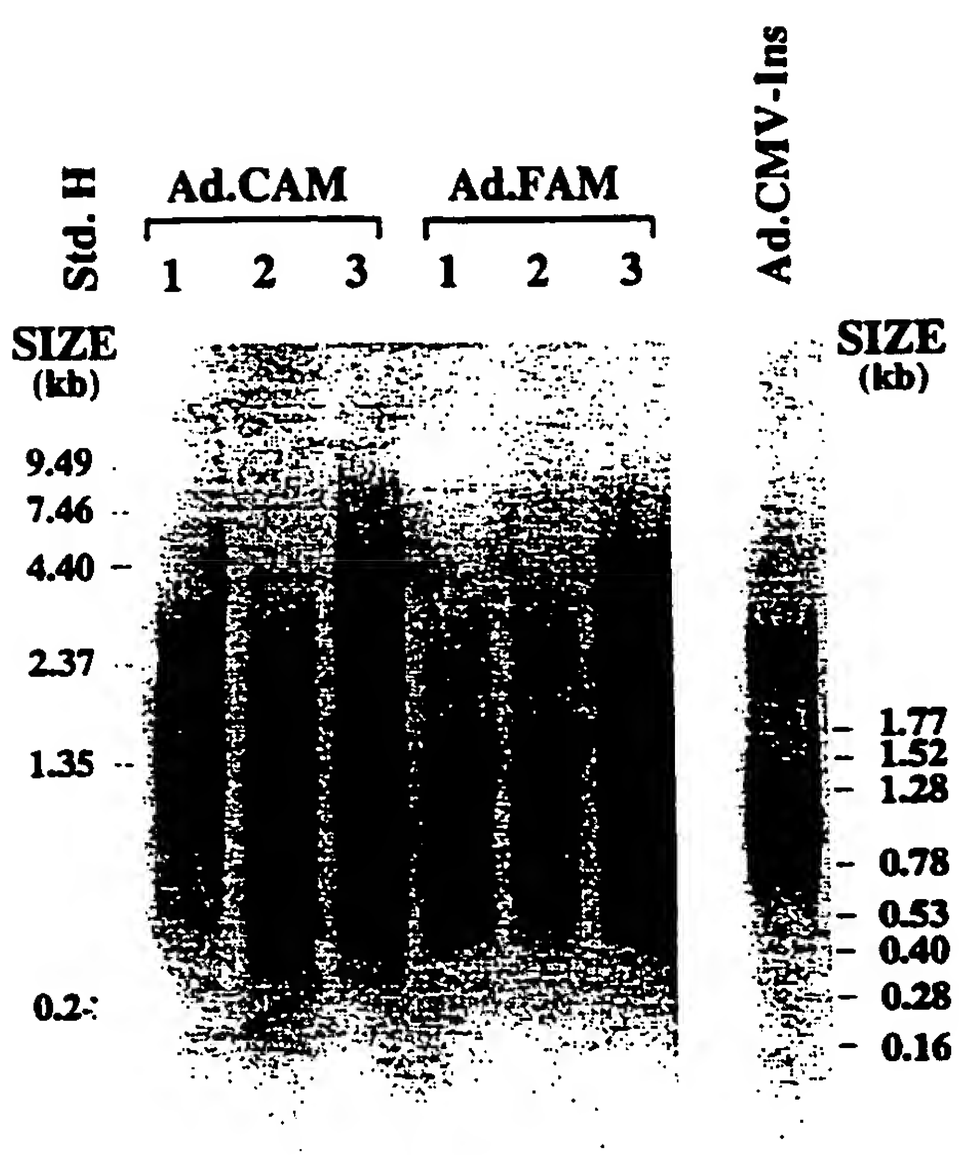
Northern
Std. H
Ad.CAM
1 2 3
Ad.FAM
1 2 3
Ad.CMV-Ins
SIZE (kb)
9.49
7.46
4.40
2.37
1.35
0.2
SIZE (kb)
1.77
1.52
1.28
0.78
0.53
0.40
0.28
0.16

FIG. 6

Northern

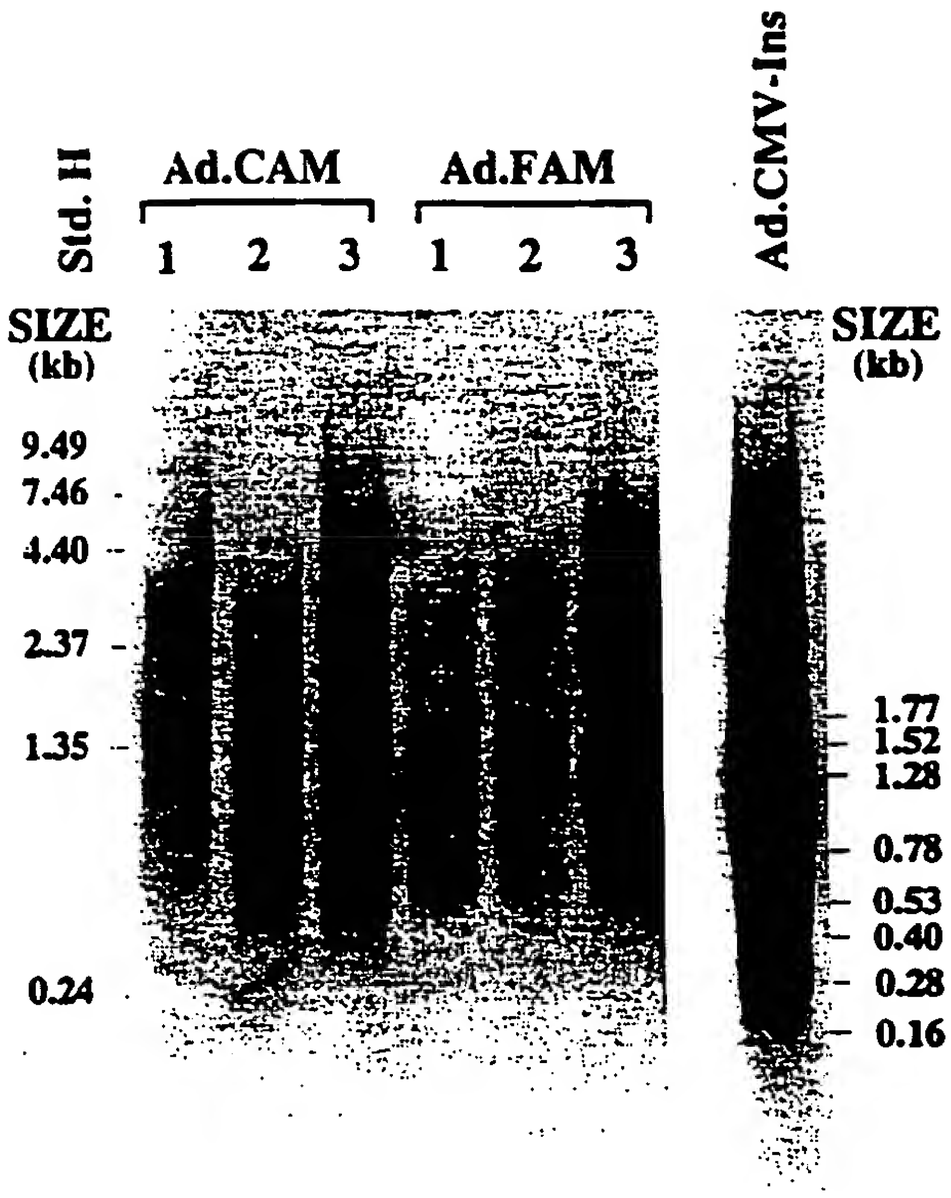

FIG. 7

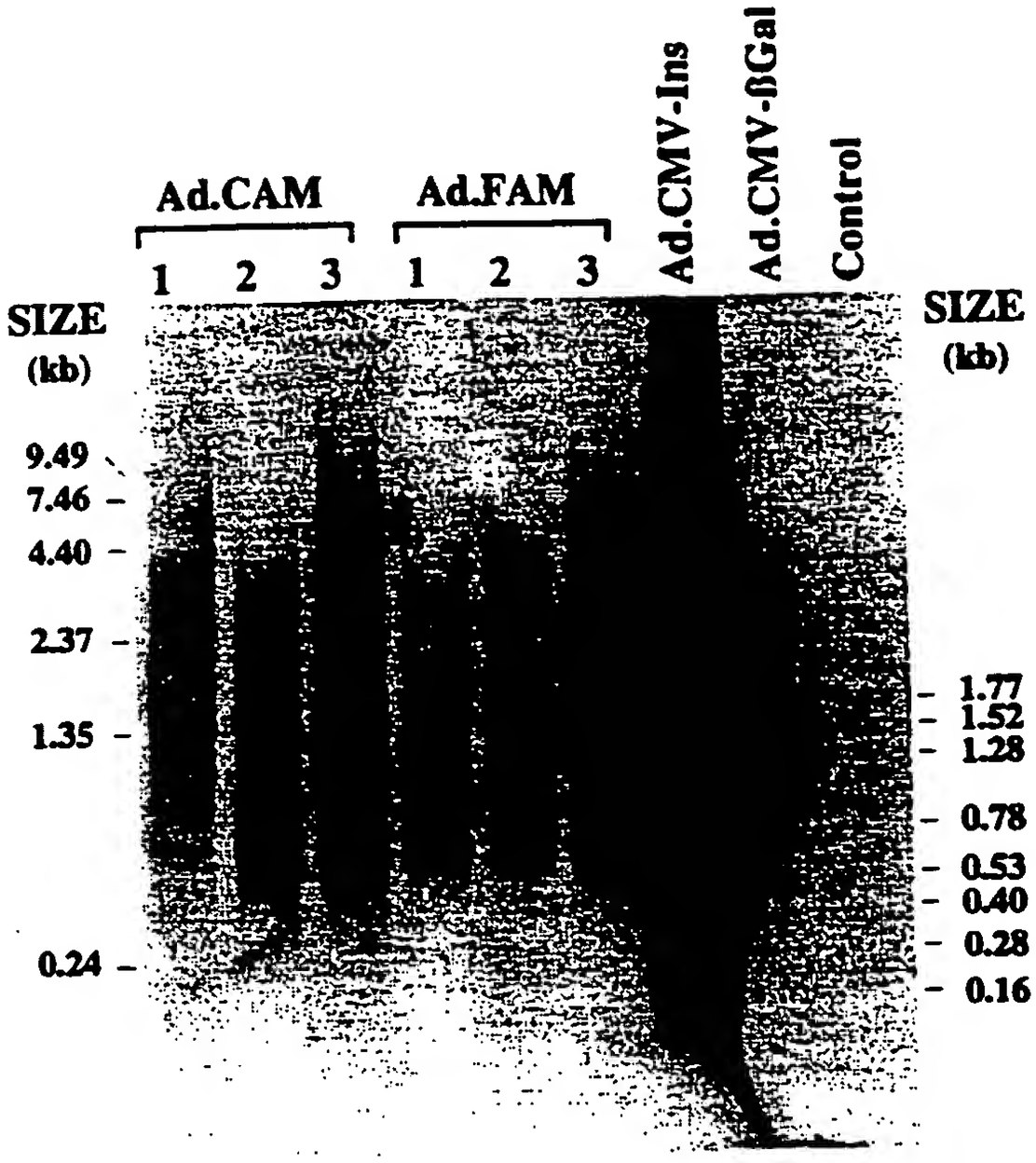
Northern
Ad.CAM
Ad.FAM
1 2 3 1 2 3
Ad.CMV-Ins
Ad.CMV-βGal
Control
SIZE (kb)
9.49
7.46
4.40
2.37
1.35
0.24
SIZE (kb)
1.77
1.52
1.28
0.78
0.53
0.40
0.28
0.16

FIG. 8

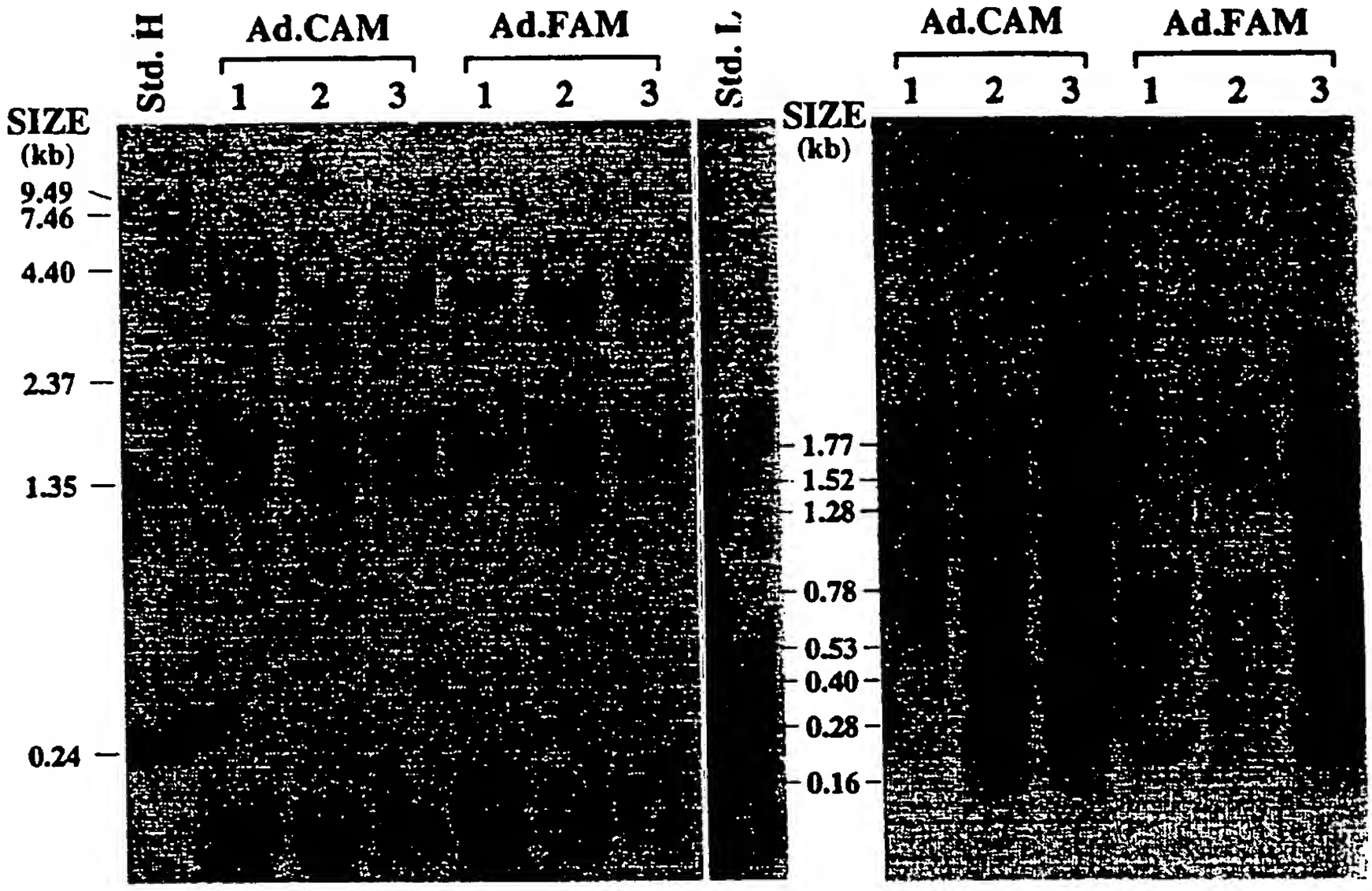
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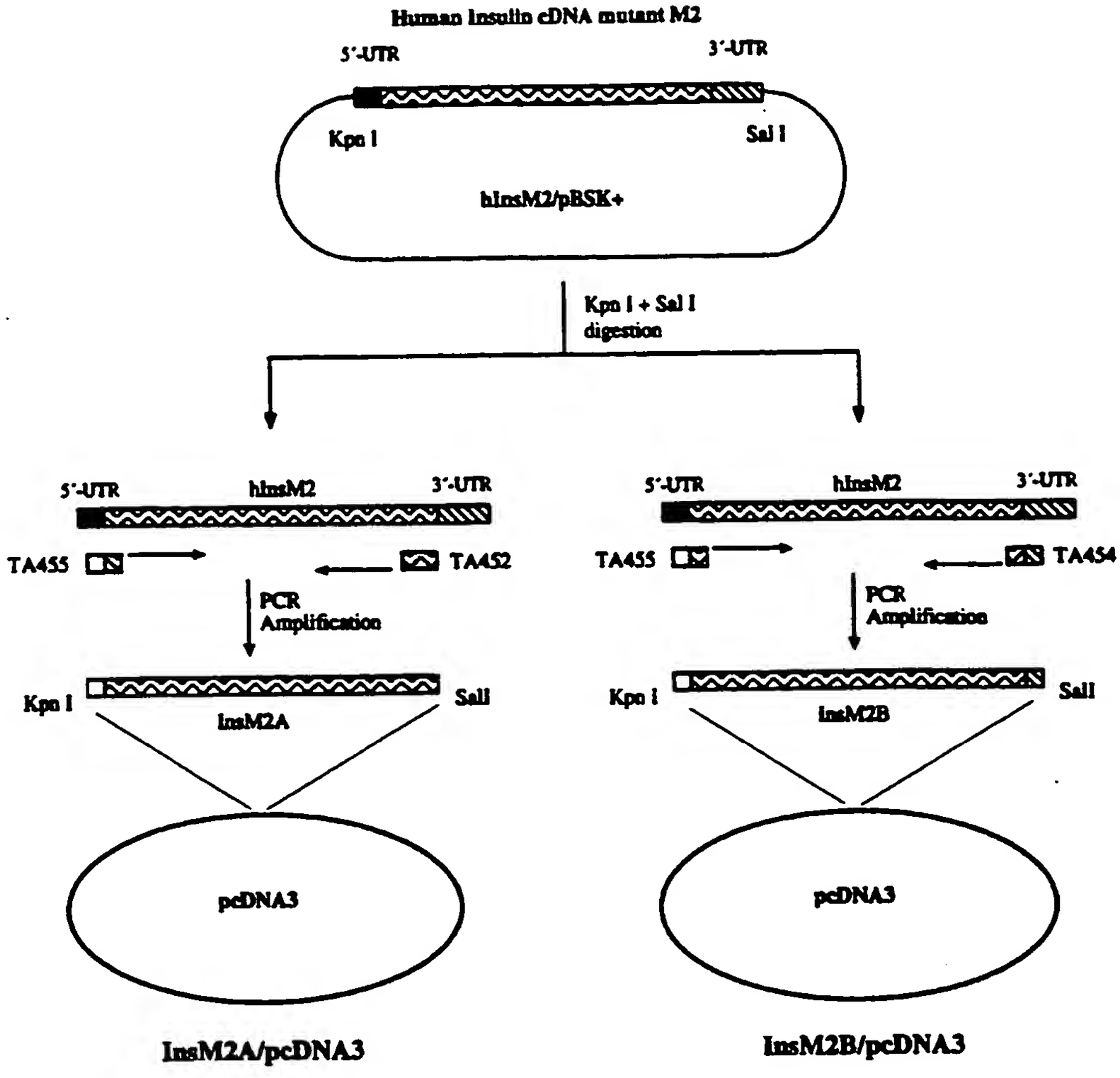
FIG. 9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5'-UTR
3'-UTR
Kpn I
Sal I
hInsM2/pBSK+
Kpn I + Sal I
digestion
5'-UTR
hInsM2
3'-UTR
TA455
TA452
PCR
Amplification
Kpn I
Sal I
InsM2A
pcDNA3
InsM2A/pcDNA3
5'-UTR
hInsM2
3'-UTR
TA455
TA454
PCR
Amplification
Kpn I
Sal I
InsM2B
pcDNA3
InsM2B/pcDNA3

FIG. 10

Insulin Released from Cos7 Cells Transfected with Recombinant Plasmids

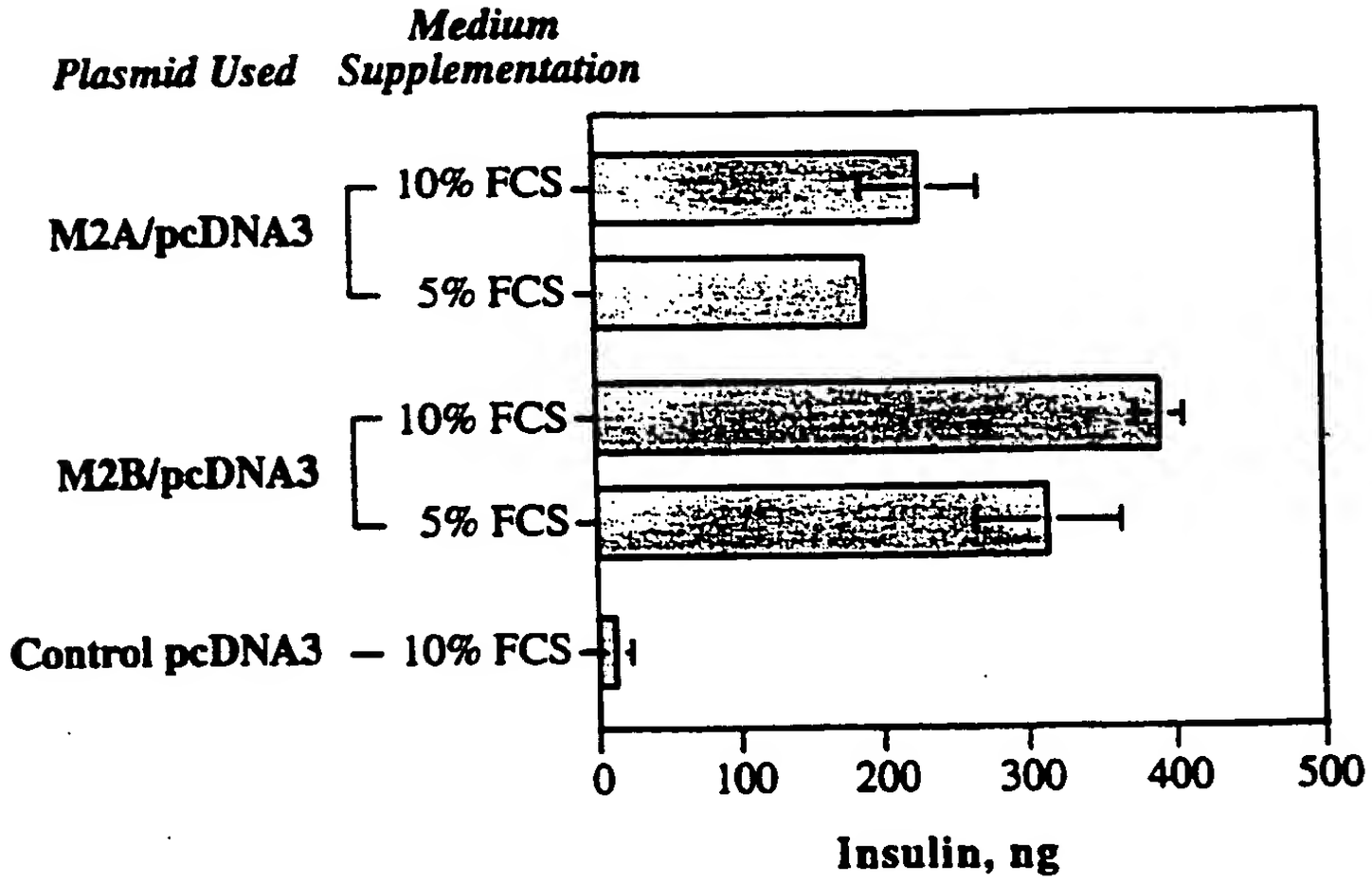

FIG. 11

Insulin Released from Cos7 Cells Transfected with Recombinant Plasmids

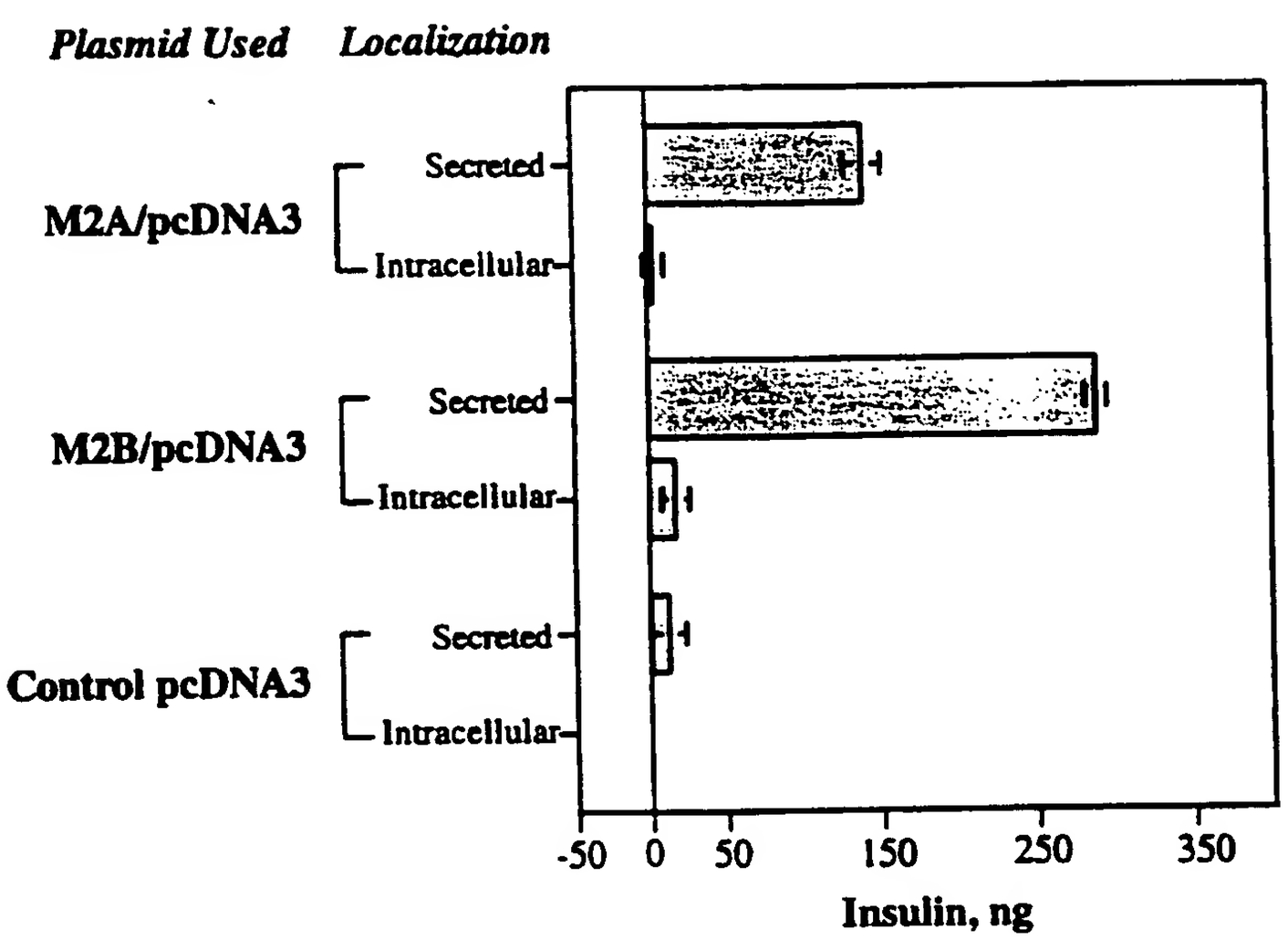

FIG. 12

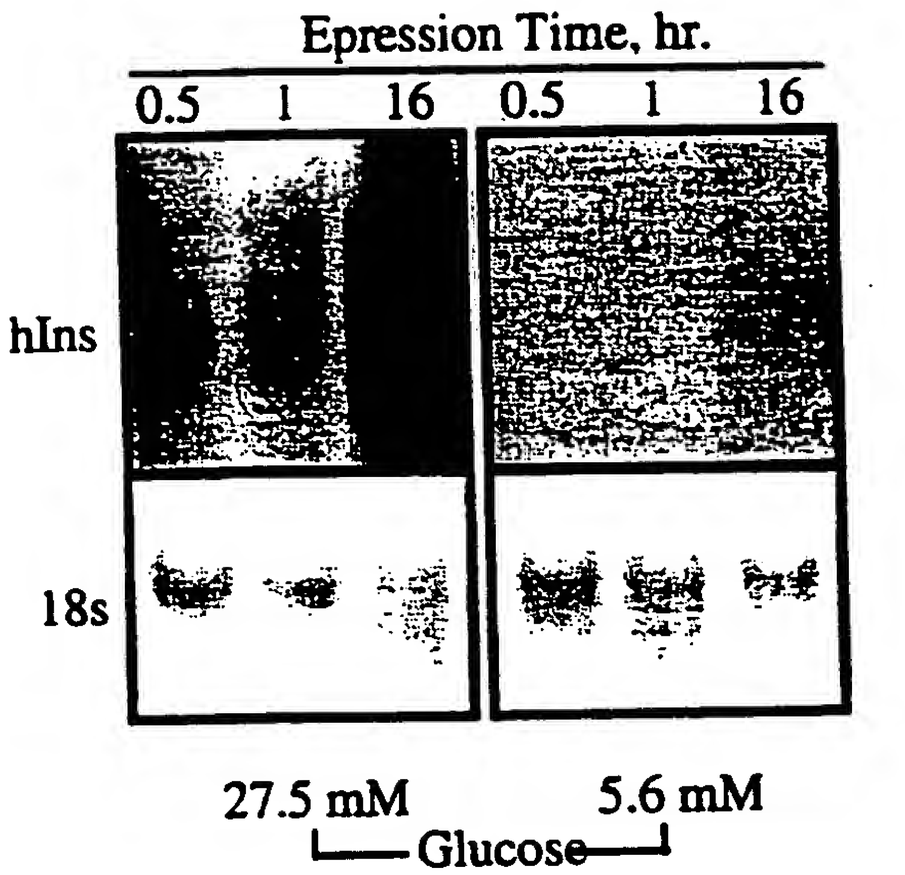

FIG. 13

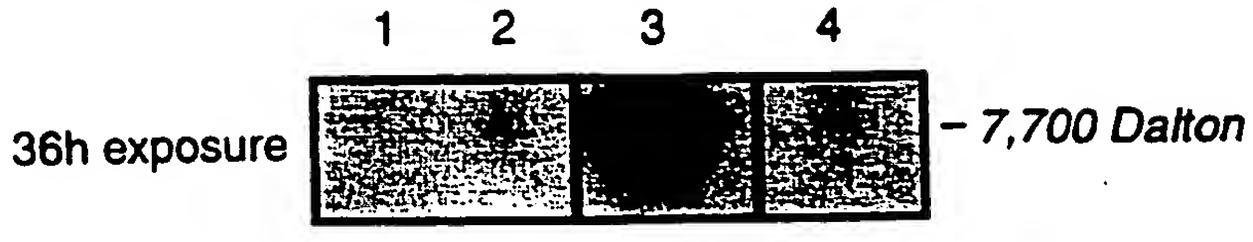

FIG. 14

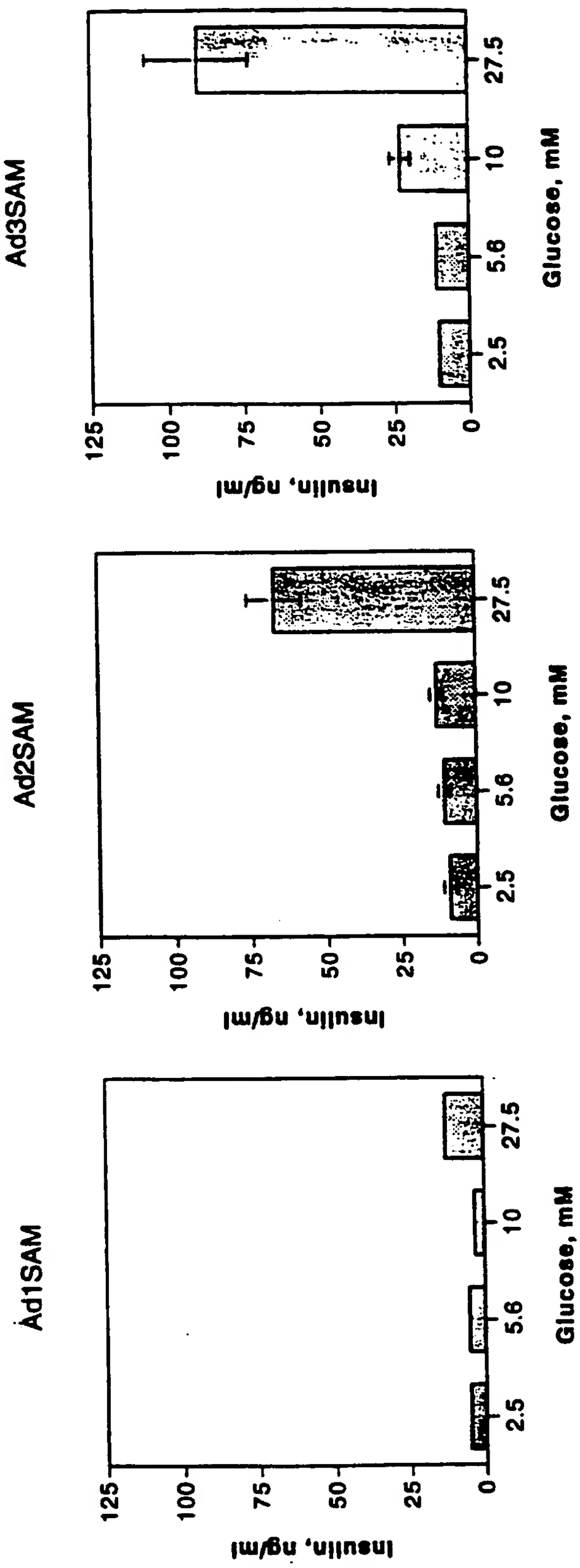

FIG. 17

FIG. 16

FIG. 15

SEQUENCE LISTING

<110> Hullett, Debra A.
Alam, Tausif
Sollinger, Hans W.
Theron, Amy

<120> Treatment of Diabetes with Synthetic Beta Cells

<130> CIP Application 96429/9003

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<151> 1997-01-21

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<213> Artificial Sequence

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and 2 arginine mutation

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gtcgac 666

INTERNATIONAL SEARCH REPORT

Intern. .ial Application No
PCT/US 98/15189

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 C12N15/85 C12N15/17 C12N15/11 C07K14/62
A61K48/00 A61K38/28 //A61P3/10,C12N15/861

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALAM ET AL: "Glucose inducible expression of human insulin in hepatocytes" ACTA DIABETOLOGICA, vol. 34, no. 2, September 1997, page 115 XP002098527 see abstract	1-4,7-9
X	MUZZIN ET AL: "Hepatic Insulin Gene Expression as Treatment for Type 1 Diabetes Mellitus in Rats" MOLECULAR ENDOCRINOLOGY, vol. 11, no. 6, 1997, pages 833-837, XP002098528 see the whole document	1,3,4, 7-9

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 1999	13/04/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Jansen, K-S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 98/15189

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIH ET AL: "Two CACGTG Motifs with Proper Spacing Dictate the Carbohydrate Regulation of Hepatic Gene Transcription" JOURNAL OF BIOLOGICAL CHEMSITRY, vol. 270, no. 37, 15 September 1995, pages 21991-21997, XP002098529 see abstract see discussion	5,6
A	VALERA ET AL: "Regulated expression of human insulin in the liver of transgenic mice corrects diabetic alterations" FASEB JOURNAL, vol. 8, 1994, pages 440-447, XP002098530 cited in the application see abstract	1-9
A	KOLODKA ET AL: "Gene therapy for diabetes mellitus in rats by hepatic expression of insulin" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 92, April 1995, pages 3293-3297, XP002098531 see abstract	1-9
E	WO 98 31397 A (WISCONSIN ALUMNI RES FOUND) 23 July 1998 see the whole document	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/15189

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 8 and 9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. .al Application No
PCT/US 98/15189

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9831397 A	23-07-1998	AU 5926998 A	07-08-1998